

**REMARKS**

Claims 23-31 are pending in this application. By this Amendment, claims 23, 26, and 30 are amended. Support for the amendments to the claims may be found, for example, in the specification at page 7, lines 1-2. No new matter is added. In view of the foregoing amendments and following remarks, reconsideration and allowance are respectfully requested.

**I. Interview**

The courtesies extended to Applicants' representative by Examiner Halverson at the interview held November 13, 2007, are appreciated. The reasons presented at the interview as warranting favorable action are incorporated into the remarks below and constitute Applicants' record of the interview.

**II. Rejection under 35 U.S.C. §112**

The Office Action rejects claim 30 under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. By this Amendment, claim 30 is amended to obviate the rejection. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

**III. Rejection under 35 U.S.C. §102(b)**

The Office Action rejects claims 23 and 31 under 35 U.S.C. §102(b) as being anticipated by Sakamoto et al., Oncol Rep, Sep-Oct 2001, 8:973-980 ("Sakamoto"). Applicants respectfully traverse the rejection.

Without conceding the propriety of the rejections, independent claim 23 is amended to more clearly recite various novel features of the claimed invention, with particular attention to the Examiner's comments. Specifically, claim 23 is amended to clarify that "the biological sample is selected from the group consisting of: blood, bone marrow, milk, cerebrospinal

fluid, urine, and effusions." Sakamoto fails to teach or suggest such a feature. Thus, Sakamoto does not anticipate claim 23. Claim 31 depends from claim 23 and, thus, also is not anticipated by Sakamoto. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested. Applicants gratefully acknowledge that Examiner Halverson agreed during the personal interview that the above-mentioned amended claim 23 is not anticipated by Sakamoto.

At the interview, Examiner Halverson raised the issue as to whether a factor that may be found in tissue would necessarily also be present in a blood, bone marrow, milk, cerebrospinal fluid, urine, or effusions.

Applicants respectfully refer the Examiner to *Kuvaja et al., Human Pathology, Vol. 37, pp. 1316-1323 (2006)* ("Kuvaja") (copy attached). Kuvaja teaches that there is an inverse correlation between the serum levels of proMMP-2-TIMP-2 complex and tissue expression of MMP-2 in the primary tumor. In particular, Kuvaja discloses that "the circulating levels of different forms of MMP-2 were finally compared with tissue expression of MMP-2 in the primary tumor. The tissue expression of MMP-2 had an inverse correlation with high serum proMMP-2-TIMP-2 complex." *See* page 1321, column 2, 3<sup>rd</sup> paragraph.

Additionally, other publications include studies that show similar results. In particular, *Shimonishi et al., Human Pathology, Vol. 32, No. 3, pp 302-310 (March 2001)* (copy attached) discloses that "Galectin-3 was frequently and strongly expressed in the cytoplasm of well differentiated ICCs, and its expression was significantly decreased and less intense or even absent in poorly differentiated ICCs." *See* page 302, first paragraph. In contrast, *Koopmann et al., Cancer, Vol. 101, No. 7, pp.1609-1615 (October 1, 2004)* ("Koopmann") (copy attached) discloses that "serum and biliary galectin-3 levels did not differ in the biliary carcinoma group relative to the control groups." *See* page 1609, first

paragraph. These articles demonstrate that tissue expression does not necessarily correlate with plasma expression.

**IV. Rejection under 35 U.S.C. §103(a)**

The Office Action rejects claims 24-27, and 29 under 35 U.S.C. §103(a) over Sakamoto, further in view of WO 97/38313 to Ts'o ("Ts'o") and Varilek et al., Am J. Physiol. 1995, 269:G445-G452 ("Varilek"). Applicants respectfully traverse the rejection.

For the reasons discussed above, Sakamoto fails to teach all of the features of independent claim 23. Notwithstanding their asserted disclosures, Ts'o and Varilek fail to cure the deficiencies of Sakamoto. Therefore, Sakamoto, Ts'o and Varilek, considered either separately or in combination, fail to teach or suggest all of the features of independent claim 23.

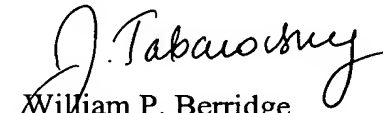
Claim 23 would not have been rendered obvious by Sakamoto, Ts'o and Varilek. Claims 24-31 variously depend from claim 23 and, thus, also would not have been rendered obvious by Sakamoto, Ts'o and Varilek. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

**V. Conclusion**

In view of the foregoing, it is respectfully submitted that this application is in condition for allowance. Favorable reconsideration and prompt allowance of the application are earnestly solicited.

Should the Examiner believe that anything further would be desirable in order to place this application in even better condition for allowance, the Examiner is invited to contact the undersigned at the telephone number set forth below.

Respectfully submitted,

  
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Attachments:

*Kuvaja et al., Human Pathology, Vol. 37, pp. 1316-1323 (2006)*  
*Shimonishi et al., Human Pathology, Vol. 32, No. 3, pp.302-310 (March 2001)*  
*Koopmann et al., Cancer, Vol. 101, No. 7, pp. 1609-1615 (October 1, 2004)*

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Original contribution

## Low serum level of pro-matrix metalloproteinase 2 correlates with aggressive behavior in breast carcinoma<sup>☆</sup>

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### Keywords:

Breast carcinoma;  
MMP-2;  
ELISA;  
Progression;  
Survival

**Summary** Malignant tumors that are capable of invading surrounding structures and metastasizing possess certain capacities to cross tissue barriers. Matrix metalloproteinases (MMPs), especially gelatinases and their inhibitor molecules, are known to affect the extracellular matrix turnover, and the proteolytic imbalance due to the abnormal expression of these enzymes eventually leads to cancer progression. This has been well documented at the tissue level. In this study, the different forms of the circulating MMP-2 have been studied in the preoperative sera of 71 patients with breast carcinoma. A quantitative enzyme-linked immunosorbent assay was performed for total proMMP-2, proMMP-2–tissue inhibitor of metalloproteinase 2 (TIMP-2) complex, and free active MMP-2. It is shown here, for the first time, that the total proMMP-2 levels in the serum correlate inversely with node positivity, high stage of the disease, and high nuclear grade of the breast tumor. An association with the levels of lower free active MMP-2 and tumor recurrence is also demonstrated. Interestingly, the tumor tissue expression of MMP-2 had an inverse correlation with proMMP-2–TIMP-2 complex levels in the serum. In conclusion, the levels of the total proMMP-2 correlate inversely with tumor burden, whereas free active MMP-2 might be associated with survival. This could indicate that the prognostic value of the circulating forms of MMP-2 is not congruent with the prognostic information obtained from tissue expression. This is further supported by the inverse correlation of the proMMP-2–TIMP-2 complex and MMP-2 tissue expression in the tumor. Therefore, the different forms of circulating metalloproteinases need to be evaluated further to explore their full potential for clinical use.

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### 1. Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases belonging to the metzincin superfamily of enzymes that control virtually all events of matrix remodeling and turnover. Today, at least 25 matrix metalloproteinase

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molecules are known. Matrix metalloproteinases 2 and 9 form a subgroup of gelatinases that have been the main interest in tumor biology [1].

Matrix metalloproteinases possess abilities to control both normal and pathologic functions of the extracellular matrix, and their own function is closely regulated at transcriptional and posttranscriptional level. At the tissue level, the natural tissue inhibitors of metalloproteinase have a significant effect in both activation and inhibition. In addition, tissue inhibitor of metalloproteinase molecules have other extracellular functions independent of MMP inhibition [2].

Gelatinases MMP-2 and MMP-9 are transcribed in the cytoplasm, packed in zymogen granules, and excreted in a latent proenzyme form that requires extracellular cleavage of the propeptide tail to become a fully active enzyme. Matrix metalloproteinase 2 has a rather complex activation mechanism that requires simultaneously the presence of the inhibitor tissue inhibitor of metalloproteinase 2 (TIMP-2) and a transmembraneous membrane-type-1 MMP molecule. Nearly all cells produce small amounts of MMP-2, and its function and activity is merely regulated posttranscriptionally, whereas the production of MMP-9 can be enhanced up to 7-fold by transcription [1].

Because of their capacity to degrade the extracellular matrix and, particularly, the basement membrane components, the gelatinolytic enzymes MMP-2 and MMP-9 have been shown to increase cancer invasion potential [3]. In clinical studies, it is well established that these gelatinases associate with cancer progression and survival in several types of malignancies [4,5]. In breast carcinoma, the association with the enhanced tissue expression of MMP-2 in the carcinoma, and a worsened survival of the patients has been presented in several publications [6-8].

The knowledge about the cross-talk between carcinomas and their microenvironment is expanding rapidly, and it is obvious that these matrix degrading enzymes play a certain role in it. It has been shown that the tissue expression of these gelatinases relates to cancer progression and survival, but the origin of these immunoreactive proteins is often discussed. More information is still needed regarding the significance of the circulating metalloproteinases in cancer progression and their possible potential as new markers in clinical decision making [9]. It is still unclear which forms of the gelatinases give the best information regarding cancer progression and survival. Therefore, we chose to investigate what information could be obtained from the different forms of the circulating MMP-2 molecules by measuring them from the preoperative sera of patients with breast carcinoma.

## 2. Materials and methods

### 2.1. Patient and control material and serum sample collection method

The patient material consisted of 71 women with primary breast carcinoma diagnosed and operated on at Oulu

University Hospital during the years 1989 to 1990. Most patients were treated solely with mastectomy and axillary dissection. Initially, none of the patients received adjuvant chemotherapy, and only 8 patients were given antiestrogen therapy with tamoxifen for 3 years after the operation. At the moment of diagnosis, the patients' median age was 55 years, ranging from 25 to 87 years. The follow-up time for each patient without events in the survival (ie, death or relapse) was 120 months at the minimum. Of the patients, 34 represented stage I disease and 37, more advanced (2A-3A) stage diseases. Fifty-three patients were node-negative, and 18 were node-positive when diagnosed. The estrogen and progesterone receptor status of the tumor was determined from 52 patients via dextran-coated charcoal assay. The menopausal status was known for 65 patients, 21 of whom were premenopausal and 44, postmenopausal. The TNM classification was done using the World Health Organization criteria and nuclear grading according to Bloom and Richardson [10,11]. The cytoplasmic expression of MMP-2 in the carcinoma cells was determined via immunohistochemistry with a monoclonal antibody that recognizes both proMMP-2 and proMMP-2-TIMP-2 complex forms. The results concerning the MMP-2 immunoreactivity and survival in node-negative patients have been published, and the method has been previously described [8]. The result for immunohistochemistry was available for 50 patients. The control group consisted of 27 healthy female volunteers.

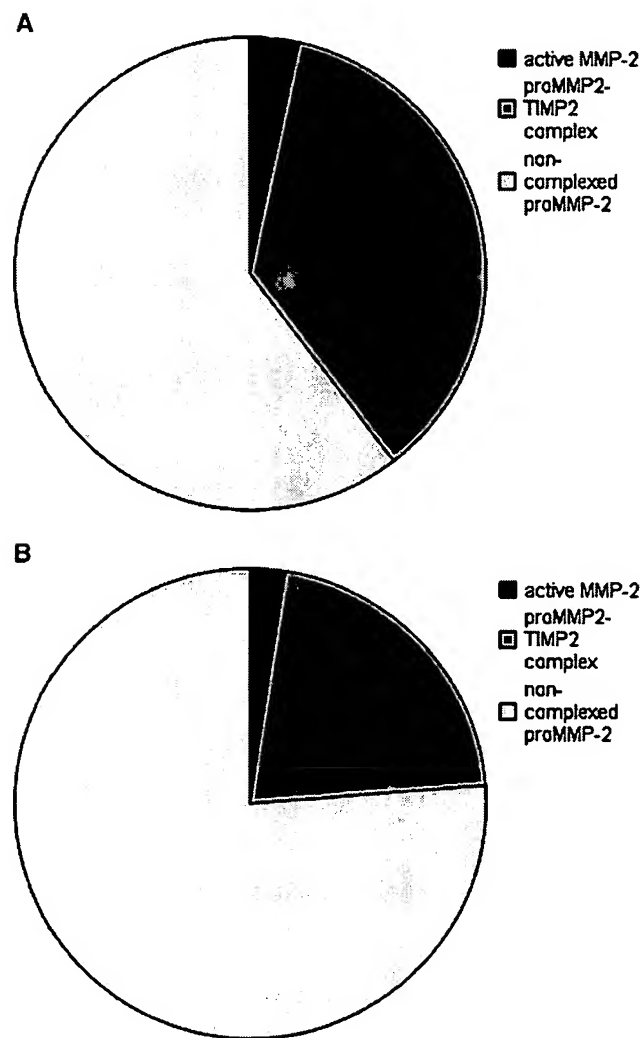
The serum samples were obtained by collecting venous blood from patients at the time of the primary diagnosis before the operation. The venous blood samples were collected in glass tubes, allowed to coagulate for 30 minutes at room temperature, and centrifugated at 3000 rpm for 10 minutes, and the serum obtained was separated, frozen, and stored at  $-20^{\circ}\text{C}$  until used. No artificial coagulation activator was used in the glass tubes that could interfere with the normal coagulation process and possibly affect the metalloproteinase levels in the serum [12].

### 2.2. Enzyme-linked immunosorbent assay method for detecting the MMP-2 levels from the serum samples

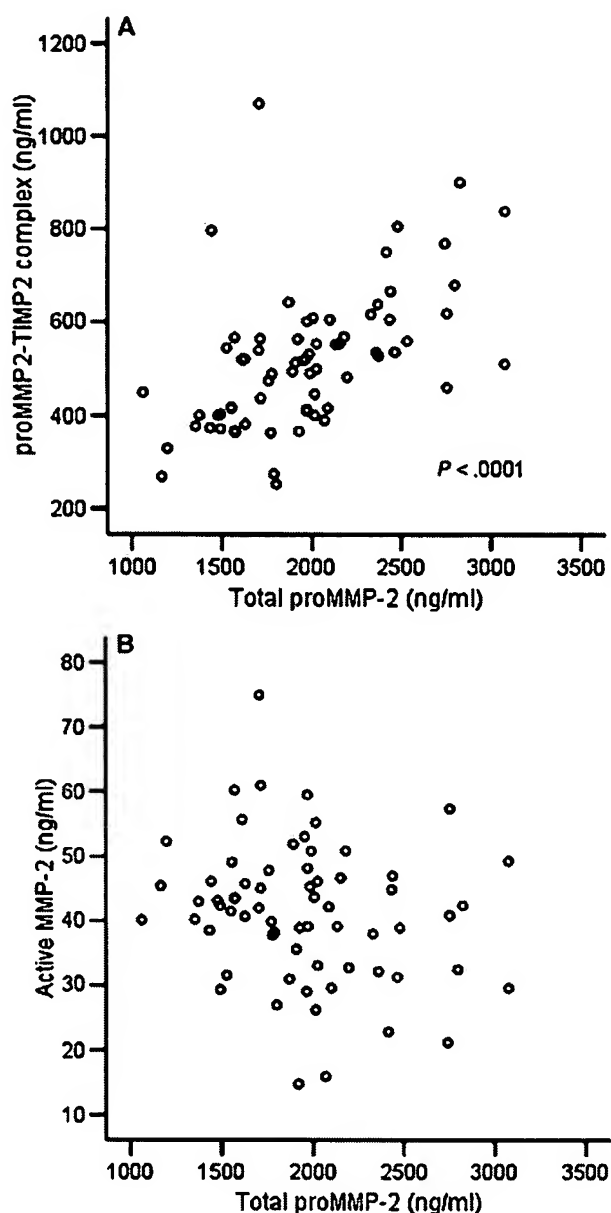
The enzyme-linked immunosorbent assay (ELISA) was used to detect the circulating total proMMP-2 levels, free active MMP-2 levels, and proMMP-2-TIMP-2 complex levels from the serum samples. A commercial assay kit (Human Biotrak ELISA system for detecting MMP-2, Amersham Biosciences, Buckinghamshire, UK) was used to detect the total and active MMP-2 levels. The assay for total proMMP-2 (RPN 2617) recognized both free proMMP-2 and proMMP-2-TIMP-2 complexes, whereas the assay used in measuring the active MMP-2 (RPN 2631) recognized only free active forms of MMP-2. The assay was conducted as recommended, following the manufacturer's instructions.

The proMMP-2-TIMP-2 complex was detected by coating the 8-well EIA/RIA strips for microtiter plates

(Coming Inc, Coming, NY) with monoclonal anti-TIMP-2 antibody (clone T2-101, SBA Sciences, Oulu, Finland). The diluted serum samples were then added, and polyclonal anti-MMP-2 (clone DB-202, SBA Sciences, Oulu, Finland) followed as the second antibody. Antichicken horseradish peroxidase enzyme (Chemicon International, Temecula, Calif) served as the enzyme conjugate, and the reaction was visualized by o-phenylenediamine dihydrochloride enzyme substrate (Sigma, Steinheim, Germany). The absorbances were read at 492-nm wavelength by Anthos Reader 2001 (Anthos Labtec Instruments, Wals, Austria). Each serum sample was run in duplicate to minimize the intraassay variation. The absorbance values for standard samples and the standard curves constructed for each assay were compared and used to minimize the interassay variation. The sensitivity of the assays for total proMMP-2, active



**Fig. 1** The proportions of the different forms of circulating MMP-2 in the serum of healthy controls (A) and patients with breast cancer (B). The serum concentrations are converted to moles; the slice areas represent molar ratios.



**Fig. 2** Correlation between the serum levels of different forms of MMP-2 in patients with breast carcinoma. A, Spearman correlation between total proMMP-2 and proMMP-2-TIMP-2 complex was 0.6. B, Pearson correlation between total proMMP-2 and active MMP-2 was  $-0.2$  (not significant).

MMP-2, and MMP-2-TIMP-2 complex were 0.37 ng/mL, 190 pg/mL, and 2 ng/mL, respectively.

### 2.3. Statistical analysis

The serum concentrations of different forms of MMP-2 were tested for correlation with clinicopathologic parameters such as tumor grade, size, stage, nodal status, estrogen/progesterone receptor status, and menopausal status by Student *t* test or analysis of variance for normally distributed continuous variables. Mann-Whitney *U* test or Kruskal-

**Table 1** Circulating total MMP-2 concentrations according to patient characteristics

Patient characteristics	No. of patients	Mean total MMP-2 concentration (ng/mL)	Significance (P)
Size of the tumor			
<2 cm	33	1964	.585
2-5 cm	24	1912	
>5 cm	5	1766	
Nodal status			
Negative	50	2044	.042
Positive	18	1793	
Stage of the tumor			
1	32	2155	.008
2	32	1826	
3	4	1774	
Nuclear grade			
1	1	2745	.047
2	7	1806	
3	9	1771	
ER status			
Negative	15	1945	.909
Positive	34	1930	
PR status			
Negative	20	1899	.620
Positive	29	1959	
Menopausal status			
Premenopausal	20	1953	.601
Postmenopausal	42	2017	

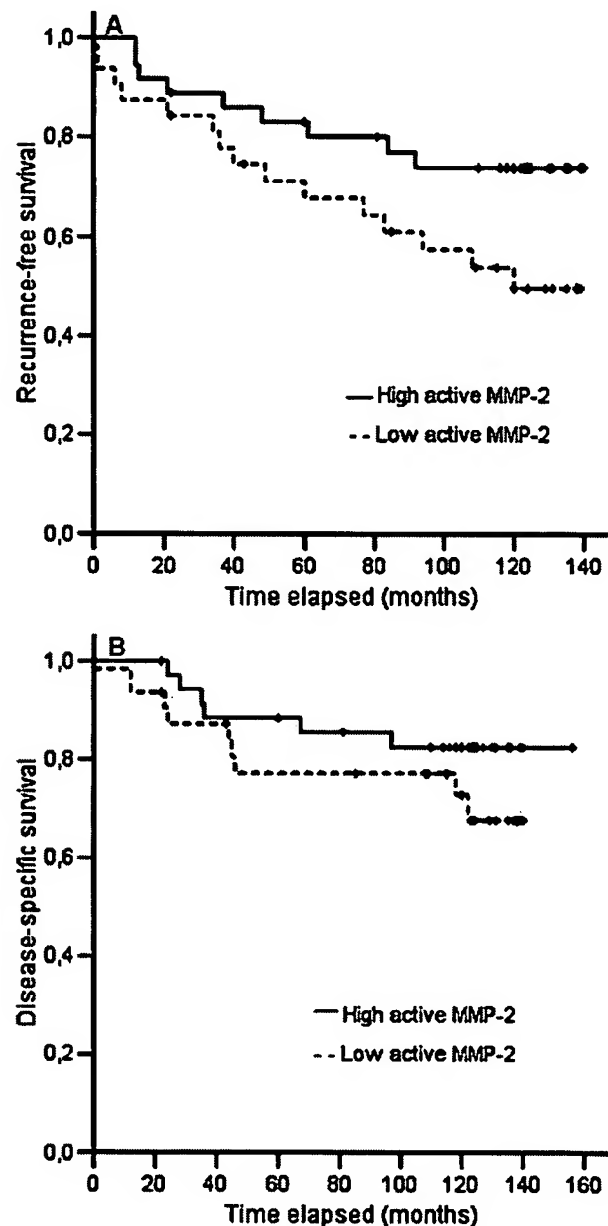
Abbreviations: ER, estrogen receptor; PR, progesterone receptor.

Wallis test was used for continuous variables otherwise normally distributed. The skewness of the distribution was determined using Kolmogorow-Smirnov test with Lilliefors significance correction. Pearson and Spearman correlations were used to test for correlation between continuous variables.  $P < .05$  was considered significant. Survival analysis was conducted using the Kaplan-Meier method for both recurrence-free and disease-specific survival. Log-rank test was used to determine the significance. Receiver operating characteristic curve was used to approximate the best cutoff values for continuous variables to conduct the Kaplan-Meier analysis. All statistics were performed using the SPSS software (SPSS, Chicago, Ill).

### 3. Results

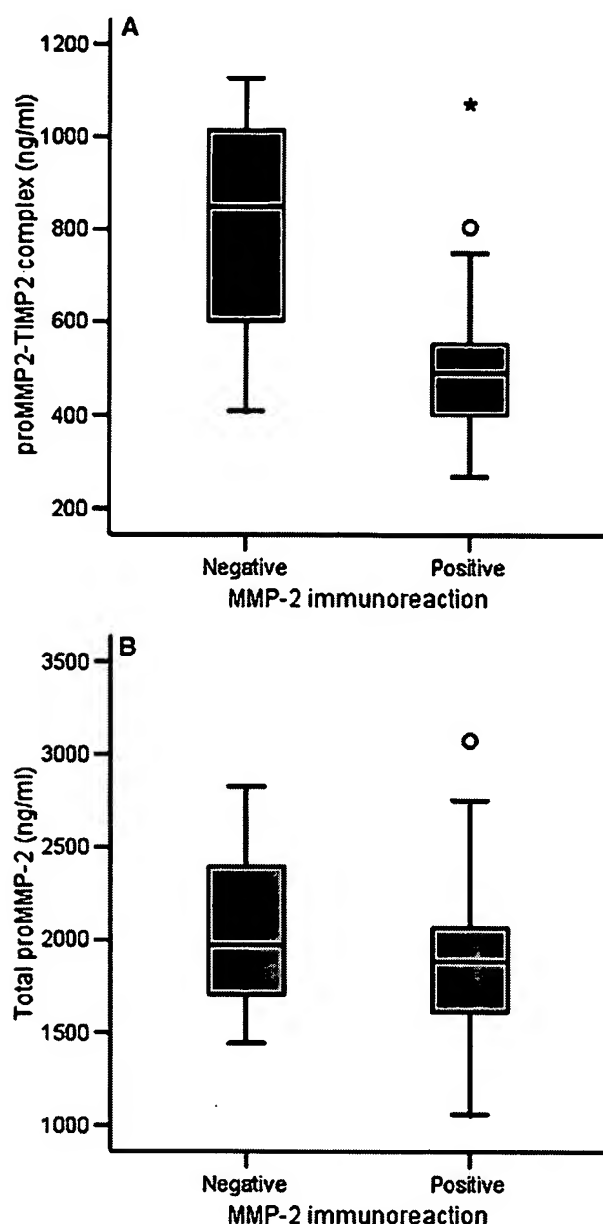
The concentrations for circulating total proMMP-2 and active MMP-2 were normally distributed for patients and controls, and their arithmetic means for proMMP-2 were 1971.6 (range, 1060.8-3075.7) and 1415.8 (range, 982.6-1938.8) ng/mL, respectively. The corresponding values for active MMP-2 were 41.2 (range, 14.8-75.0) and 42.6 (range,

37.1-49.9) ng/mL. The distribution of the concentrations of the proMMP-2-TIMP-2 complex was found to be skew for patients, and its median value was 520.6 ng/mL (range, 253.2-1126.8 ng/mL). For the controls, the distribution was normal, with a mean value of 625.2 ng/mL (range, 464.5-886.5 ng/mL). The patients had significantly higher serum content of the total proMMP-2 than controls ( $P < .001$ ), whereas the controls had higher proMMP-2-TIMP-2 complex levels ( $P < .001$ ). This indicates a higher proportion of proMMP-2 in the complex form in the control



**Fig. 3** Tumor recurrence and disease-specific survival according to high (>40.7 ng/mL) or low ( $\leq 40.7$  ng/mL) levels of active MMP-2. A, The recurrence-free survival according to high ( $n = 36$ ) or low ( $n = 32$ ) active MMP-2 ( $P = .058$ ). B, The disease-specific survival according to high or low active MMP-2 levels (not significant).





**Fig. 4** The levels of proMMP-2-TIMP-2 complex and total proMMP-2 according to tissue expression of MMP-2. A, The mean rank for proMMP-2-TIMP-2 complex was 39.5 in the group of patients with negative ( $n = 4$ ) and 24.3 with positive ( $n = 46$ ) immunoreaction in the primary tumor ( $P = .045$ ). B, The mean value for total proMMP-2 in the group of negative immunoreaction was 2079.9 and 1880.4 ng/mL in the positive group (not significant).

group (Fig. 1A and B), although the ranges for patient and control groups were overlapping for both proMMP-2 and proMMP-2-TIMP-2. No differences were found for active MMP-2 between patients and controls in the analyses.

In the patient material, the levels of circulating total proMMP-2 correlated with the levels of proMMP-2-TIMP-2 complex ( $P < .0001$ ,  $R = 0.52$ ) (Fig. 2A), but not with the active MMP-2 levels ( $P = .1$ ,  $R = -0.2$ ) (Fig. 2B).

When classifying patients into groups according to clinicopathologic parameters, it was found that the patients with higher tumor burden had significantly lower levels of circulating total proMMP-2. A significant result was obtained for nodal status ( $P = .042$ ), stage of the disease ( $P = .008$ ), and nuclear grade ( $P = .047$ ) (Table 1). A similar trend, although not significant, was also present according to the size of the tumor (Table 1). For active MMP-2 or proMMP-2-TIMP-2 complex, no significant correlations with clinicopathologic parameters were found (data not shown).

In survival analyses, it was noticed that the patients with lower levels of circulating active MMP-2 had relapses more often than those with higher level ( $P = .058$ ). The best cutoff value for dividing the patients into groups of high or low levels was found to be 40.7 ng/mL. Kaplan-Meier analyses were conducted according to this cutoff value. At 10 years of follow-up, only 50% of the patients with low levels of circulating active MMP-2 were relapse-free, compared with 74% in the patient group with higher serum levels ( $P = .058$ ) (Fig. 3A). There was also a difference, although not a significant one, in the disease-specific survival according to this division. In the patient group of low levels of active MMP-2, there were more deaths by the disease, the survival being 68% at 10 years of follow-up. In the group of high levels, the survival was better (83% at 10 years of follow-up) (Fig. 3B). For total proMMP-2 or proMMP-2-TIMP-2 complex, no associations with recurrence-free or disease-specific survival were found (data not shown).

The circulating levels of different forms of MMP-2 were finally compared with tissue expression of MMP-2 in the primary tumor. The tissue expression of MMP-2 had an inverse correlation with high serum proMMP-2-TIMP-2 complex; the levels were significantly higher in the group of negative immunoreaction ( $P = .045$ ) (Fig. 4A). For total proMMP-2, the trend was similar although not significant (Fig. 4B). For active MMP-2, no differences were found according to the MMP-2 immunoreactivity (data not shown).

## 4. Discussion

Previously, a few studies have been published on the significance of the circulating MMP-2 measured in serum samples of patients with breast cancer, comparing the level with disease progression. However, this study is, to the best of our knowledge, the first one to discuss the relevance of the different forms of circulating MMP-2 in breast cancer.

The levels of MMP-2 in the serum of patients with breast cancer have shown great variation in previous reports. In studies where ELISA analysis was used to determine the serum content of MMP-2, the mean values have varied from 5.25 [13] to 649.3 ng/mL [14], although it was not mentioned which form of MMP-2 was measured in the former work. Studies using zymographic detection methods have reported a mean concentration of 320.1 [15] and a

median concentration of 950.3 ng/mL [16] in breast carcinoma. These reflect the levels of the noncomplexed proMMP-2 content of the serum because the bands in the zymograms corresponded to 72 kd. Our levels of the total proMMP-2 were higher than those reported earlier, but this is reasonable because the assay registered all proMMP-2 molecules and both free and proMMP-2-TIMP-2 complexes.

For the first time, an inverse correlation between high tumor burden (positive nodal status, higher stage of the disease, and higher nuclear grade) and serum level of the total proMMP-2 is shown in breast carcinoma. Previously, La Rocca et al [15] found a negative correlation with estrogen receptor expression, but no other correlations with size of the tumor, nodal status, or the stage were found. A negative association was reported in their study between the nuclear grade of the tumor and the level of proMMP-2 in the serum of 80 patients with breast cancer, which is congruent with our results. Previously, it has been shown conflictly that the high levels of MMP-2 in the sera of patients with breast cancer correlate with advanced stage and higher lymph node status [14], as well as with an adverse prognosis [13]. However, in these studies, it was not explained which form of MMP-2 was determined, and the levels of serum MMP-2 differ remarkably in the 2 studies.

An association with the levels of circulating active MMP-2 and tumor recurrence and survival is demonstrated in this study. It is notable that the active MMP-2 might implicate different aspects than the total proMMP-2 because there was no correlation between their serum levels. It could be possible that the total proMMP-2 and proMMP-2-TIMP-2 complex levels in the preoperative serum could reflect the underlying conditions of the disease, whereas the active MMP-2 might be more closely linked to cancer progression and survival.

At the tissue level, the active MMP-2 is used in proteolysis and cell migration occurring in the process of metastatic spread [17]. Lower serum levels in the group of more frequent recurrence could reflect the higher efficacy of the usage of active MMP-2 in the carcinoma tissue. The actual activation process for soluble proMMP-2 taking place on the cell surface requires the presence of membrane-type MMP and of TIMP-2 simultaneously. Recent studies have demonstrated that additional molecules, such as integrins and extracellular MMP inducers, are involved in the process by presenting and linking the soluble MMPs to the cell surface for activation [17-19]. Their expression might therefore be crucial to the usage of MMPs in the proteolysis. In a similar manner, more efficient localization of circulating soluble proMMP-2 via extracellular MMP inducers, integrins, or membrane-type MMPs to the cell surface, leading to enhanced activation of proMMP-2, could explain the correlation between lower total proMMP-2 levels and more advanced disease.

The healthy controls had lower levels of total proMMP-2 and higher levels of the proMMP-2-TIMP-2 complex than the patients. This might support the hypothesis that the carcinoma, either by itself or by a host response, is able to

produce increased amounts of proMMP-2 as a circulating reserve, but the activation process is the determining factor for cancer invasiveness that includes trapping and linking proMMP-2 molecules to the cell surface. No differences were found between healthy controls and patients in terms of active MMP-2 in the serum, but this could also be because of the short half-life of the active MMP-2. However, this finding should be interpreted with caution because the control group was not age-standardized, and age seems to affect the levels of the metalloproteinases and their inhibitors in the blood.

Other explanatory models for the partly puzzling result could be found in the recently proposed connections between MMPs and apoptosis. In a recently published work, Simian et al [20] studied the activities of MMPs in a hormone-induced mammary tumor regression model. They found that an increase in the activities of MMP-2, MMP-3, and MMP-9 coincided with a peak in the apoptotic activity in the tumor regression process. They propose that a loss of contact with the basement membrane due to proteolysis leads to increase in the apoptotic signaling, and MMPs might therefore induce apoptosis. A similar result exists for melanoma [21]. This could explain the result of high proMMP-2 and high active MMP-2 relating to less advanced disease and enhanced survival via increased apoptosis in the carcinoma tissue.

This is also the first study to show an inverse correlation between the serum levels of the proMMP-2-TIMP-2 complex and tissue expression of MMP-2 in the primary tumor. This is interesting because the origin of MMP-2 in the malignant tissue has been extensively studied and discussed. Studies on clinical material report the localization of MMP-2 by immunohistochemistry or enhanced expression of MMP-2 mRNA to be in the tumor cells [22,23] and in the peritumoral area [23,24], whereas a recent study on mouse mammary tumors found the expression of MMP-2 mRNA to be mainly located in the stromal cells outside the actual tumor area [25]. As it was discussed earlier, there may be several mechanisms by which the tumor tissue might enhance the production of these molecules in the surrounding tissue and then anchor them to the cell surface for further usage. The inverse correlation, albeit partial, could also support the hypothesis that the molecule is actually produced outside the tumor area. The inverse correlation could reflect a situation where some types of malignant cells might have the potential to catch the MMP molecules from the circulation, and larger and more advanced tumors would therefore have higher tissue expression and, thus, lower levels of circulating MMP-2.

Few studies exist on the clinical relevance of preoperative circulating MMP-2 in other malignancies. In colorectal cancer, Oberg et al [26] found no correlation between preoperative serum-free MMP-2 level and clinical stage, but the patients in the highest quartile of serum MMP-2 had shorter survival, compared with patients with lower levels of

MMP-2. Recently, Langenskiöld et al [27] showed a positive correlation of proMMP-2 (both free and complexed with TIMP-2) plasma levels with lymph node status. However, here, the tumor-class (T) demonstrated an inverse correlation with proMMP-2 so that lower T-class tumors had elevated levels of proMMP-2 in comparison with T4 tumors. In this work, no correlation with survival was found. Waas et al [28] studied the levels of proMMP-2 in the plasma via ELISA and zymography. They found an inverse correlation with plasma MMP-2 levels and lymph node involvement, size of the tumor, and stage, and patients with colorectal cancer had significantly lower plasma levels of proMMP-2 than did healthy controls. A correlation between high stage and high serum proMMP-2 in lung cancer was shown by Sasaki et al [29], but they failed to show any correlation with survival. In a study by Hoikkala et al [30], a correlation of the higher serum levels of proMMP-2-TIMP-2 complex with lower stage of the disease and enhanced survival was demonstrated.

The results, so far, on the significance of the circulating metalloproteinases have shown great variation and proven themselves conflicting in almost all carcinoma subtypes. It appears that the most crucial process in the MMP-mediated carcinoma progression is their activation. It has been shown in many studies that at the tissue level, the enhanced MMP-2 activity correlates with carcinoma progression and poor survival [18,19,31,32].

We show here, for the first time, that low circulating levels of MMP-2 in the preoperative serum of patients with breast cancer are associated with a higher tumor burden and tumor recurrence and are therefore not congruent with its prognostic value in tissue expression in breast carcinoma. This is further supported by the partial inverse correlation between serum MMP-2 and tissue expression. It is evident that different forms of MMP-2 reflect different capacities in breast cancer progression, and therefore, they need to be studied further to explore their full potential as clinical indicator molecules.

## Acknowledgments

The authors thank Mrs Anne Bisi for help in laboratory procedures.

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# Expression of Endogenous Galectin-1 and Galectin-3 in Intrahepatic Cholangiocarcinoma

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Galectins, a family of  $\beta$ -galactoside-binding animal lectins, might be involved in tumor progression. In this study, the expression patterns of galectin-1 and -3 were examined immunohistochemically in intrahepatic cholangiocarcinoma (ICC), with emphasis on its development and progression as well as its histopathologic features, by use of samples of normal intrahepatic bile duct ( $n = 20$ ), biliary epithelial dysplasia ( $n = 15$ ), ICC ( $n = 40$ ), and a cholangiocarcinoma cell line, CCKS1. In normal intrahepatic bile ducts, galectin-3 was constitutively but weakly expressed, whereas galectin-1 was not expressed. In hepatolithiasis, biliary epithelial dysplasia was strongly positive for galectin-3 but negative for galectin-1. Galectin-3 was frequently and strongly expressed in the cytoplasm of well-differentiated ICCs, and its expression was significantly decreased and less intense or even absent in poorly differentiated ICCs. Galectin-1 was expressed in carcinoma cells in ICC, and its incidence and extent were correlated with histologic dedifferentiation of ICC. Proliferative cell nuclear antigen (PCNA) labeling index (LI) was higher in ICC cases positive for galectin-1 than in those that were negative. Galectin-1 was strongly expressed in cancerous stroma of ICC, and this stromal expression was related to histologic dedifferentiation of

ICC. In the carcinoma cell line CCKS1, galectin-1 and -3 were expressed in the cytoplasm of carcinoma cells, and galectin-1 was additionally detected in the culture medium. These results suggest that galectin-1 was newly expressed on carcinoma cells of ICC, and its overexpression seems to be associated with neoplastic progression and proliferative activities, and the expression of galectin-1 in cancerous stroma may also be related to the progression of ICC. Galectin-3 expression in epithelial cells is up-regulated in the preneoplastic and early neoplastic stages of ICC, although galectin-3 tends to disappear at later stages of ICC. HUM PATHOL 32:302-310. Copyright © 2001 by W.B. Saunders Company

**Key words:** biliary epithelial dysplasia, galectin, hepatolithiasis, immunohistochemistry, intrahepatic bile duct, intrahepatic cholangiocarcinoma.

**Abbreviations:** Ig, immunoglobulin; ECM, extracellular matrix; ICC, intrahepatic cholangiocarcinoma; EVG, elastica-van-Gieson; DAB, 3,3'-diaminobenzidine tetrahydrochloride; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; LI, labeling index; SDS, sodium dodecyl sulfate.

Galectins are a family of  $\beta$ -galactoside-binding animal lectins.<sup>1-7</sup> At present, more than 10 galectins have been characterized in mammals.<sup>8</sup> Many studies have been done on galectin-1, the prototype of this gene family, and on galectin-3 which was identified as the Mac2 macrophage marker and also named as immunoglobulin (Ig) E-binding protein CBP35.<sup>7</sup> Galectin-1 and -3 have molecular weights of 14.5 and 26 to 30, respectively, and they show both intracellular and extracellular localization.<sup>7</sup> Although the precise functions of galectins-1 and -3 remain speculative in individual tissues or diseases, there is evidence that they play a role in cell-to-cell adhesion,<sup>9,10</sup> cell-to-extracellular matrix (ECM) interactions,<sup>11-13</sup> cellular proliferation,<sup>14-17</sup> differentiation,<sup>12,18,19</sup> pre-mRNA splicing,<sup>20</sup> and apoptosis.<sup>21,22</sup>

The significance of the expression of both galectin-1 and galectin-3 is now being evaluated in many neoplasms. Studies on human epithelial tumors such as colorectal, thyroid, gastric, ovarian, and breast carcinomas have shown that the expression pattern of galectins is related to the tumor stages or progression, invasion, and metastasis behavior.<sup>23-31</sup> In fact, expression patterns of galectin-1 and/or -3 have been shown to be a valuable tumor marker for prediction of metastasis and poor prognosis in some malignant tumors.<sup>23-28</sup>

As for the hepatobiliary system, there have been a few reports on the expression of galectins in normal and pathologic livers.<sup>32,33</sup> For example, Hsu et al<sup>32</sup> recently reported that galectin-3 expression was induced in cirrhotic liver, particularly in the peripheral portions of regenerative nodules, as well as in hepatocellular carcinoma. They speculated that deregulated expression of galectin-3 can result in tumor transformation and invasiveness or confer a propensity for survival in tumor cells of hepatocyte lineage.<sup>32</sup> However, the expression of galectins and their significance have not been explored in the biliary tree.

In this study, we immunohistochemically surveyed the expression of galectin-1 and -3 in the intrahepatic biliary tree and intrahepatic cholangiocarcinoma (ICC) with an emphasis on neoplastic transformation and

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progression. Expression and secretion of galectin-1 and -3 were also examined in the human cholangiocarcinoma cell line CCKS1.<sup>34,35</sup>

## MATERIALS AND METHODS

### Classification of the Intrahepatic Biliary Tree

The intrahepatic biliary tree was classified as intrahepatic large and small bile ducts.<sup>36,37</sup> The former are grossly visible and characterized by the presence of a fibrous ductal wall and accompanying peribiliary glands. They correspond to the right and left hepatic ducts, segmental ducts, area ducts, and their first to second branches. The small bile ducts that are recognizable under a microscope are classified as septal and interlobular bile ducts and bile ductules according to their size and location in the portal tracts.<sup>36,37</sup>

### Definition of Biliary Epithelial Dysplasia

Biliary epithelial dysplasia is defined as epithelial cells showing multilayering, piled-up nuclei, and micropapillary projection into the ductal lumen. Cytologically, the dysplastic epithelial cells have an increased nucleocytoplasmic ratio, a partial loss of nuclear polarity, and nuclear hyperchromasia. However, these atypical features are mild and inadequate for diagnosing malignancy. This lesion is preferentially found in the lining epithelia of intrahepatic large bile duct.

### Tissue Specimens

The human livers and ICC specimens used in this study were from the surgical files of our laboratories and affiliated hospitals. We collected 40 cases of ICC; surgically resected specimens (mean age 66 years; range 44 to 87 years; 18 male, 22 female) that were subdivided into 15 hilar types and 25 peripheral types, according to the method of Okuda et al.<sup>38</sup> In all 40 cases the ICCs were adenocarcinomas, with 18 well-differentiated ICCs (including 4 papillary adenocarcinomas), 10 moderately differentiated ICCs, and 12 poorly differentiated ICCs. Liver specimens from 15 cases of hepatolithiasis with biliary epithelial dysplasia and hyperplasia (mean age 64 years; range 39 to 89 years; 4 male, 11 female), all surgical, were also obtained. Biliary epithelial dysplasia in hepatolithiasis is proposed to be a precursor lesion of ICC.<sup>39-41</sup> Twenty normal liver samples containing non-neoplastic intrahepatic bile ducts were selected from normal areas of surgical specimens of ICC, more than 5 cm away from the foci of carcinoma. These normal liver specimens were used for investigation of intrahepatic large and septal bile ducts, peribiliary glands, interlobular bile ducts, and bile ductules.

All tissue specimens were immediately fixed in 10% neutral buffered formalin and embedded in paraffin. Several 4- $\mu$ m-thick histologic sections were cut from each paraffin-embedded block. Two of them were stained with hematoxylin and eosin for histologic evaluation and with elastica-van-Gieson (EVG), respectively. Vascular invasion and lymphatic permeation were assessed on EVG-stained sections and graded as none, mild, or severe. The remaining sections were processed for immunohistochemical analysis.

Evaluation of tumor size and extrahepatic metastasis was performed by ultrasound sonography, computed tomography, and magnetic resonance imaging as well as by macroscopic and microscopic examination in surgical cases. In

total, 11 of 40 ICC cases had extrahepatic metastasis, the sites of which were lymph nodes.

### Cholangiocarcinoma Cell Line CCKS1

This cell line was established from the abdominal metastatic foci of an intrahepatic cholangiocarcinoma (60-year-old man).<sup>34,35</sup> The original histology was of moderately differentiated adenocarcinoma. CCKS1 proliferated, forming a tumor mass, after heterotransplantation to nude mice, and the transplanted tumor had the same histology as the original peritoneal metastasis.<sup>34,35</sup>

**Preparation of Culture Medium.** Equal volumes of Ham F12 medium (Flow Laboratories, Irvine, Scotland) and Dulbecco modified Eagle medium (Flow Laboratories), supplemented with 10% heat-inactivated fetal calf serum (Gibco, New York, NY), were used as culture medium; 200 U/mL of penicillin G and 100 mg/mL of streptomycin (both from Meiji Seika, Tokyo, Japan) were added to prevent bacterial contamination.

**Culture of CCKS1 Cells.** CCKS1 cells were dispersed in culture medium and seeded in Lab-TEK chamber slides containing 2 wells (Nunc, Naperville, IL) coated with type I collagen (Nittazertan Corp, Osaka, Japan). The culture was maintained for 2 weeks in an incubator under a 5% CO<sub>2</sub> atmosphere at 37°C until the cultured CCKS1 cells reached confluence. Next, the slides were fixed in 4% paraformaldehyde (PFA; Wako Chemical, Osaka, Japan) for 10 minutes at room temperature, air-dried, and stored at -80°C until use.

### Preparation of Antibodies Against Galectin-1 and -3

Recombinant lectins, human 14-kd and 30-kd  $\beta$ -galactoside-binding lectins (galectin-1 and -3), were produced in an *Escherichia coli* system, and polyclonal antibodies against them were prepared in rabbit.<sup>42,43</sup>

### Immunohistochemistry

Staining of histologic tissue sections and CCKS1 cells cultured on chamber slides was performed using the EnVision+ system (Dako; Glostrup, Denmark).<sup>44</sup> After deparaffinization and rehydration, and following blocking of endogenous peroxidase and incubation in normal goat serum (diluted 1:10; Vector Lab, Burlingame, CA) for 20 minutes, the sections were incubated overnight at 4°C with rabbit anti-14-kd galectin (galectin-1) antiserum<sup>42</sup> and rabbit anti-30-kd galectin (galectin-3) antiserum<sup>43</sup> and subsequently incubated at room temperature for 1 hour with goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase (for galectin-1) or peroxidase (for galectin-3) labeled dextran polymer (Envision+). The sections for galectin-1 and -3 were visualized with Vector red (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Company, St Louis, MO) as the chromogen, respectively. The sections were counterstained with hematoxylin. No immunohistochemical staining was obtained when nonimmune serum or phosphate-buffered saline (PBS) was used instead of the primary antibody. To confirm the specificity of the galectin staining, we also carried out preabsorption and competition experiments. Some sections were preincubated with the antigen at a concentration of 50 mg/mL for 30 minutes and then incubated with the antibody in the presence of the antigen (50 mg/mL).

Proliferating cell nuclear antigen (PCNA) and S-100

were also examined immunohistochemically using monoclonal antibody against PCNA (Novocastra Labo, Newcastle, England), rabbit anti-cow S-100 (Dako), and the EnVision+ system. The latter antibody is known to cross-react strongly with human S-100 A and B.

Vector Red, which was used for visualization of reaction products, sheds red fluorescence under a fluorescence microscope with a rhodamine filter system. To analyze the distribution of galectin-1 and -3, we examined the slides of CCKS1 cells with a LSM410 laser confocal microscope (Carl Zeiss, Gottingen, Germany) using an argon laser (514 nm) at a scanning speed of 8.65 seconds and photographed them with a Polaroid camera (Camera Back L-III; Avio, Tokyo, Japan).

### Semiquantitative Evaluation of Immunohistochemistry

In galectin-1 and -3 immunostainings, the immunohistochemical results in ICCs were semiquantitatively classified into the following 4 groups: 0, no staining; 1+, <25% of cells positive; 2+, 25% to 65% positive; and 3+, >65% positive. These scores were applied to both epithelial and stromal components of each kind of lesion. Stained carcinoma cells were compared with the adjacent normal intrahepatic bile ducts when these were present.

A PCNA-labeling index (LI) for the evaluation of cell-proliferative activity was calculated by counting positive cells among 1,000 nuclei of ICC and 500 nuclei of biliary epithelial cells in normal livers and hepatolithiasis. Perineural invasion was also assessed on S-100-immunostained sections and was graded as none, mild, or severe in individual cases.

### Protein Extraction and Immunoblotting

Subconfluent monolayers of cultured CCKS1 cells and the culture medium were obtained separately from the culture plates. The isolated cultured cells and culture medium were homogenized separately in 1.5 volumes of a solution containing 0.1 mol/L lactose and 4 mmol/L 2-mercaptoethanol in PBS (pH 7.2) at 4°C. Each suspension was transferred to a microfuge tube and centrifuged at 1,200 rpm for 1 minute at 4°C. The supernatant fraction was mixed with an equal volume of a sample buffer (pH 6.8, 0.25 mol/L Tris-HCl, 2% sodium dodecyl sulfate [SDS], 10% 2-mercaptoethanol, 30% glycerol, and 0.01% bromophenol blue) and boiled for 6 minutes. Protein in each sample was separated by 15% to 25% gradient SDS polyacrylamide gel electrophoresis (Multi Gel; Daiichi Pure Chemicals, Tokyo, Japan) at 40 mA for 60 minutes. The gels were then equilibrated for 30 minutes in transfer buffer (50 mmol/L Tris, 40 mmol/L glycine, 0.04% SDS, and 15% methanol). Proteins were electrophoretically transferred onto polyvinylidene difluoride membrane (Immunobilon PVDF transfer membrane; Daiichi Pure Chemicals). Membranes were incubated with 3% bovine serum albumin in PBS at 4°C overnight and subsequently incubated with rabbit anti-14-kD galectin (galectin-1) and/or rabbit anti-30-kD galectin (galectin-3) antiserum for 1 hour at room temperature. After 3 washes in T-PBS (0.03% Tween 20 in PBS), the membranes were treated with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer (EnVision+). After 3 washes in T-PBS, the protein band was detected by immersion in DAB (0.2 mg/mL)-H<sub>2</sub>O<sub>2</sub> (0.075%) at room temperature. No immunohistochemical staining was obtained when nonimmune serum or PBS was used instead of the primary antibody.

### Statistical Analysis

Kruskal-Wallis rank test, Spearman rank-correlation test, Mann-Whitney *U* test, and Student *t* test were used, with a significance level of  $P < .05$ .

## RESULTS

### Normal Livers

**Galectin-1.** Intrahepatic bile ducts and hepatocytes were negative for galectin-1 (Fig 1A). Vascular endothelial cells of the portal vein and hepatic arterial branches and nerve bundles in the portal tracts were positive for galectin-1. Connective tissue in portal tracts and parenchyma was weakly positive or negative for galectin-1.

**Galectin-3.** Intrahepatic bile ducts were homogeneously but weakly positive. The expression patterns were almost cytoplasmic with membranous accentuation and rarely intranuclear staining (Fig 2A). In addition, endothelial cells of vessels, nerve bundles, and mononuclear cells in the periductal connective tissue and portal tracts were positive for galectin-3. Hepatocytes were frequently positive for galectin-3 with a cytoplasmic granular pattern.

### Hepatolithiasis Alone

**Galectin-1.** Hyperplastic and dysplastic biliary epithelial cells as well as peribiliary glands in 15 cases of hepatolithiasis were negative for galectin-1 (Fig 1B, Table 1). However, the mesenchymal cells and vasculature and extracellular matrices in the fibrously thickened bile duct wall of the stone-bearing bile ducts were positive for galectin-1 (Table 2).

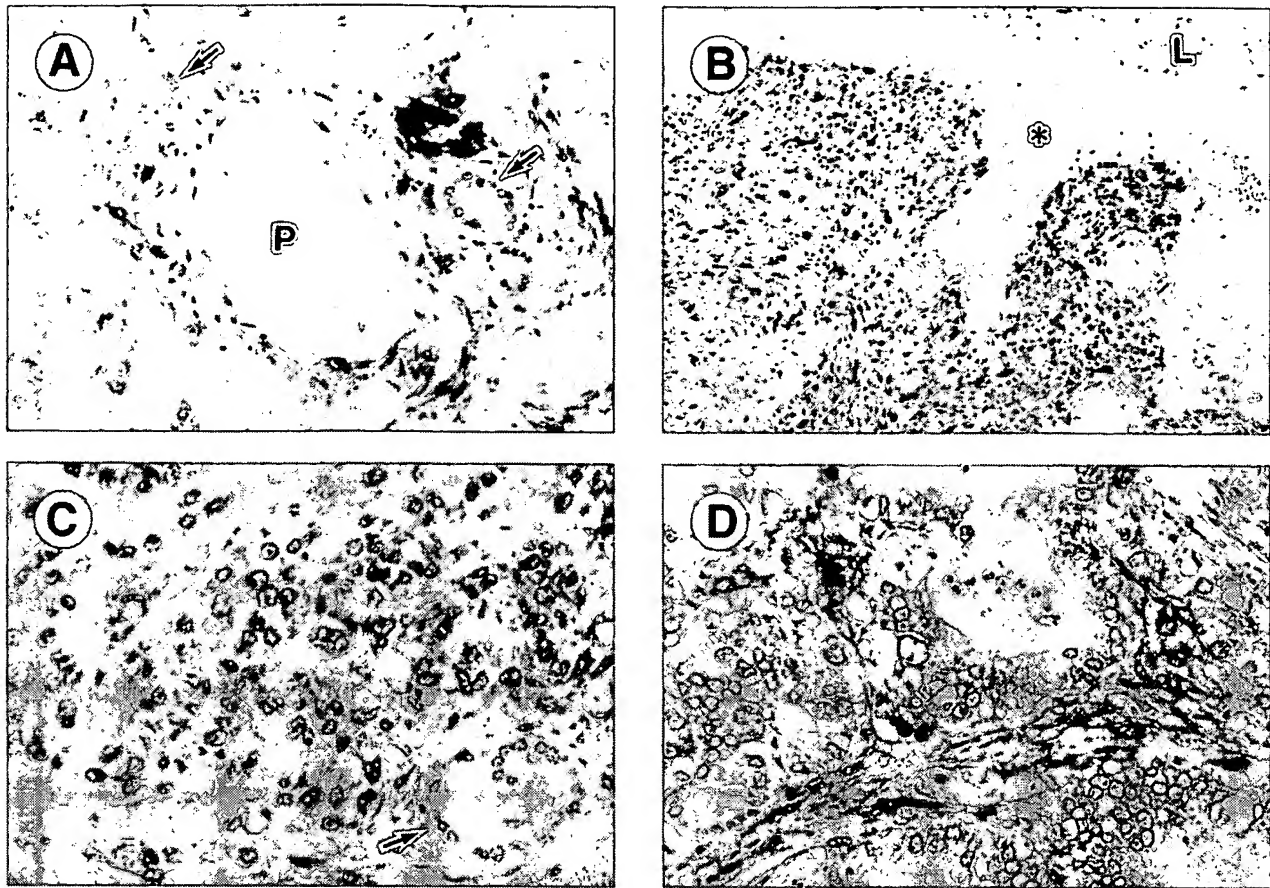
**Galectin-3.** Almost all biliary epithelial dysplasia and peribiliary glands around the stone-bearing bile ducts were intensely and homogeneously positive, and the staining intensity was stronger than that in the normal bile ducts (Fig 2B). The hyperplastic biliary epithelia without dysplasia were weakly and homogeneously positive. Hepatocytes were negative or weakly positive.

### ICC

**Galectin-1.** In 29 of 40 ICC cases (73%), carcinoma cells were positive for galectin-1 (Table 1). In the positive cases, galectin-1 was expressed in the cytoplasm and less frequently at the cell membrane (Fig 1C). It was expressed more frequently in poorly differentiated ICCs than in well-differentiated and moderately differentiated ICCs ( $P < .05$ ; Table 1). The expression of galectin-1 was significantly correlated with tumor size ( $>4$  cm;  $P < .05$ ) and tended to be higher in peripheral ICCs than in hilar ICCs (Table 3). In addition, the expression of galectin-1 was more frequent and intense in the ICC cases with vascular invasion, lymphatic permeation, and lymphnode metastasis than in those without, except for perineural invasion ( $P < .05$ ; Table 3).

ICCs also showed an extensive stromal galectin-1





**FIGURE 1.** Expression of galectin-1 in normal adult liver (A) and in hepatolithiasis (B) in ICC (C,D). (A) The expression of galectin-1 is negative in normal bile ducts (arrows) and is weakly positive in stromal cells in the portal tract, such as endothelial cells. P, portal vein. (B) Biliary epithelial dysplasia and proliferated peribiliary glands in hepatolithiasis are negative for galectin-1. The mesenchymal cells, fibroblasts, and vasculatures in the fibrously thickened bile duct wall of stone-bearing bile ducts show strong galectin-1 expression. L, bile duct lumen; \*Biliary epithelial dysplasia. (C) ICC shows strong galectin-1 expression in carcinoma cells and none in normal bile ducts (arrow). (D) ICC shows strong galectin-1 expression in carcinoma cells and stromal cells. (Vector Red and hematoxylin; Original magnifications: A,  $\times 400$ ; B,  $\times 200$ ; C,  $\times 400$ ; D,  $\times 400$ .)

expression (stromal cells and extracellular matrix) compared with normal portal tract and parenchyma (Fig 1D). This stromal expression of galectin-1 was frequent and extensive in 55% of ICCs, and this was significantly correlated with histologic dedifferentiation of ICC ( $P < .05$ ; Table 2). Stromal galectin-1 expression in ICCs did not correlate with the location or size of carcinomas, lymphatic permeation, and lymph node metastasis. However, this expression was significantly correlated with the perineural and vascular invasion ( $P < .05$ ; data not shown).

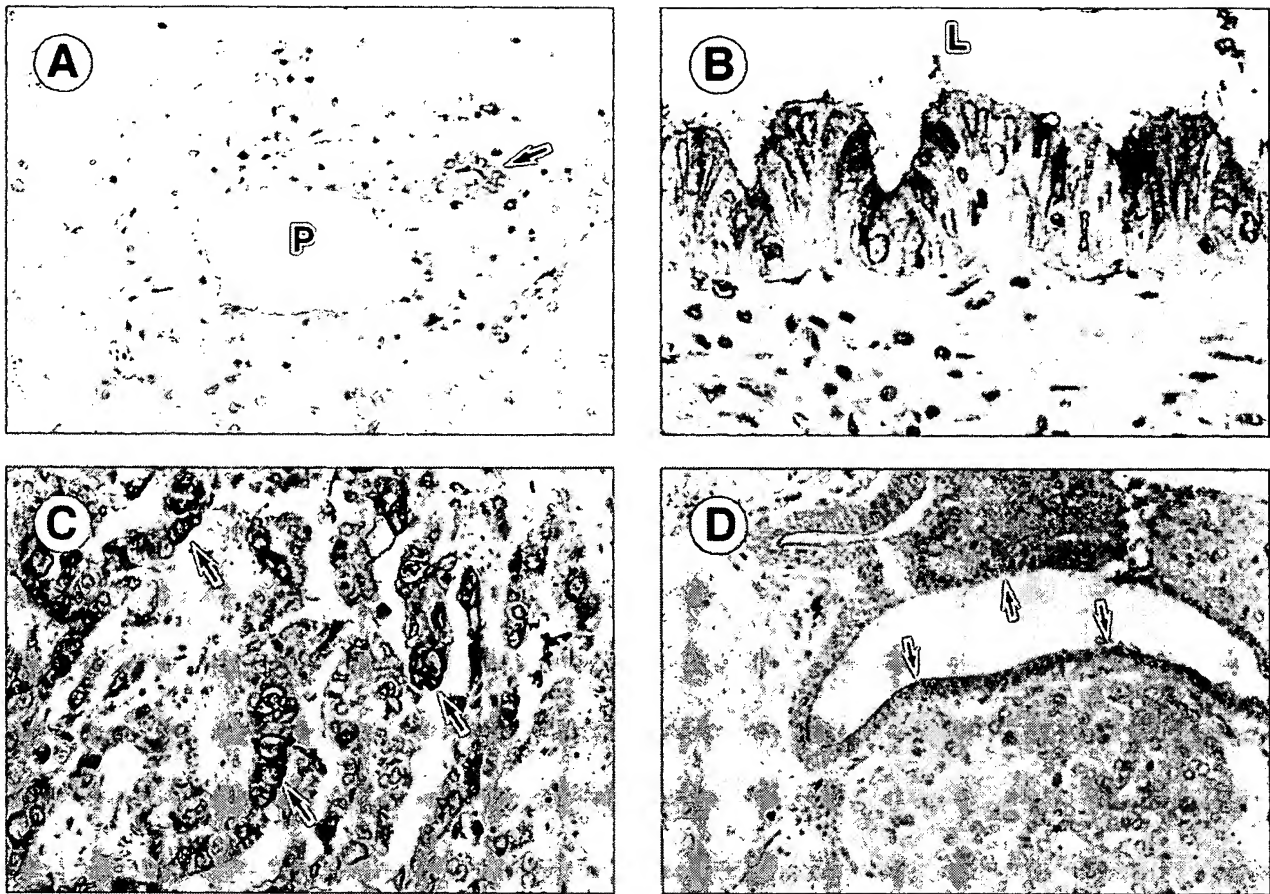
**Galectin-3.** Carcinoma cells of 37 of 40 ICC cases (93%) were positive for galectin-3 (Table 4). Galectin-3 was expressed in the cytoplasm of carcinoma cells but rare in the nucleus. The intensity and frequency of galectin-3 expression was stronger in well-differentiated ICCs, including papillary ICCs, than in normal bile ducts ( $P < .05$ ; Fig. 2C, Table 4). However, its expression was rather infrequent and weak in moderately and poorly differentiated ICCs compared with well-differen-

tiated ICCs ( $P < .05$ ; Fig 2D, Table 4). There was a tendency for the expression of galectin-3 to be stronger in papillary ICCs and well-differentiated areas showing intraluminal growth than in less well-differentiated areas in the same ICC liver when different grade areas were present in a single liver. There were no significant differences in the correlation between the occurrence of perineural and vascular invasion and lymphatic permeation and the expression of galectin-3; however, galectin-3 expression was significantly correlated with the absence of lymphnode metastasis and tumor size ( $< 4$  cm;  $P < .05$ ; Table 5). In addition, there was no significant correlation between tumor location and expression of galectin-3 (Table 5).

#### PCNA-LI

There were significant differences in PCNA-LI among normal bile ducts (LI  $0.84 \pm 0.6$ ), biliary epi-





**FIGURE 2.** Expression of galectin-3 in normal adult liver (A) and in hepatolithiasis (B) in ICC (C,D). (A) Normal bile duct (arrow) shows weak galectin-3 expression. P, portal vein. (B) Biliary epithelial dysplasia shows strong galectin-3 expression. L, bile duct lumen. (C) ICC shows strong galectin-3 expression in carcinoma cells (arrows). (D) ICC shows weak or negative galectin-3 expression but normal bile duct (arrows). (DAB and hematoxylin; Original magnifications: A,  $\times 400$ ; B,  $\times 640$ ; C,  $\times 400$ ; D,  $\times 400$ .)

thelial dysplasia in hepatolithiasis ( $9.2 \pm 8.3$ ), and carcinoma cells in ICCs ( $13.6 \pm 9.2$ ;  $P < .05$ ). There were also significant differences in PCNA-LI among well-differentiated ( $7.5 \pm 2.4$ ), moderately differentiated

( $14.4 \pm 8.4$ ), and poorly differentiated ICCs ( $18.11 \pm 1.11$ ;  $P < .05$ ). The PCNA-LI was significantly higher in ICC cases positive for galectin-1 ( $16.4 \pm 10.8$ ) than in ICC cases negative for galectin-1 ( $10.7 \pm 6.1$ ;  $P < .05$ ). No significant differences were noted between PCNA-LI and the expression of galectin-3 in ICC.

**TABLE 1.** Biliary Epithelial Expression of Galectin-1 in Normal Intrahepatic Bile Duct, Biliary Dysplasia, and ICC

	No. of cases	Galectin-1 Expression*			
		-	1+	2+	3+
Normal intrahepatic bile duct	20	20	0	0	0
Biliary dysplasia	15	15	0	0	0
ICC†	40	11	16	11	2
Well differentiated	18	7	8	3	0
Moderately differentiated	10	4	4	1	1
Poorly differentiated	12	0	4	7	1

\* -, negative; 1+, <25% of cells positive; 2+, 25% to 65% positive; 3+, >65% positive.

† Spearman rank-correlation test for histologic differentiation of ICC,  $P < .01$ .

#### CCKS1 Cells Cultured on Lab-TEK Chamber Slides

Galectin-1 and galectin-3 were expressed in the cytoplasm of cultured CCKS1 cells. Staining was diffuse (Fig 3).

#### Detection of Galectin-1 and Galectin-3 by Immunoblot Analysis

Immunoblot analysis of extracts from the human cholangiocarcinoma cell line CCKS1 and its culture medium showed that CCKS1 cells expressed a high level of both 14-kd (galectin-1) and 30-kd (galectin-3) protein, whereas the culture medium expressed only 14-kd protein (Fig 4). Only a single band was detected

**TABLE 2.** Stromal Expression of Galectin-1 in Normal Intrahepatic Bile Duct, Biliary Dysplasia, and ICC

	No. of cases	Galectin-1 Expression*			
		-	1+	2+	3+
Normal intrahepatic bile duct	20	20	0	0	0
Biliary dysplasia	15	0	0	0	15
ICC†	40	2	16	14	8
Well differentiated	18	2	9	7	0
Moderately differentiated	10	0	3	5	2
Poorly differentiated	12	0	4	2	6

\* -, negative; 1+, <25% of cells positive; 2+, 25% to 65% positive; 3+, >65% positive.

† Spearman rank-correlation test for histologic differentiation of ICC,  $P < .01$ .

for each galectin, verifying the specificity of the antibodies for their respective galectins.

## DISCUSSION

Recent studies show that galectin-1 and -3 are involved in early and advanced neoplastic events and in tumor progression.<sup>23-32</sup> However, their expression is variable depending on tumor type and on cancer models. For example, Xu et al<sup>26</sup> report that malignant thyroid tumors of epithelial cell origin and metastatic lymph nodes of papillary carcinomas express galectin-1 at high levels, and neither benign thyroid tumors nor normal adjacent tissues express it at all. Raz et al<sup>5</sup> report

**TABLE 3.** Relationship Between Biliary Epithelial Galectin-1 Expression and Pathologic Features in 40 Cases of ICC

	No. of cases	Galectin-1 Expression*			
		-	1+	2+	3+
Type					
Hilar	15	6	7	1	1
Peripheral	25	5	9	10	1
Size					
< 4 cm	19	7	9	3	0†
≥ 4 cm	21	4	7	8	2†
Perineural invasion					
Negative	24	9	9	5	1
Positive	16	2	7	6	1
Vascular invasion					
Negative	19	9	7	3	0†
Positive	21	2	9	8	2†
Lymphatic permeation					
Negative	13	7	5	0	1†
Positive	27	4	11	11	1†
Lymph node metastasis					
Negative	29	11	11	7	0†
Positive	11	0	5	4	2†

\* -, negative; 1+ <25% of cells positive; 2+, 25% to 65% positive; 3+, >65% positive.

† Mann-Whitney  $U$  test,  $P < .05$ .

**TABLE 4.** Biliary Epithelial Expression of Galectin-3 in Normal Intrahepatic Bile Duct, Biliary Dysplasia, and ICC

	No. of cases	Galectin-3 Expression			
		-	1+	2+	3+
Normal intrahepatic bile duct	20	0	0	6	14
Biliary dysplasia	15	0	0	0	15
ICC†	40	5	9	16	10
Well differentiated	18	1	1	7	9
Moderately differentiated	10	0	4	6	0
Poorly differentiated	12	4	4	3	1

\* -, negative; 1+, <25% of cells positive; 2+, 25% to 65% positive; 3+, >65% positive.

† Spearman rank-correlation test for histologic differentiation of ICC,  $P < .01$ .

that metastatic tumor cells express higher levels of galectin-1 than nonmetastatic cells. As for galectin-3, a positive correlation with tumor progression has been reported for colon carcinomas,<sup>23</sup> thyroid carcinomas,<sup>26</sup> and anaplastic large-cell lymphomas,<sup>45</sup> and a negative correlation has been described for gastric carcinoma,<sup>27</sup> breast carcinoma,<sup>31</sup> ovarian carcinoma,<sup>30</sup> and colon carcinoma.<sup>29</sup>

In this study, we first disclosed that galectin-1 was expressed in carcinoma cells of 73% of ICC cases, while normal and dysplastic biliary epithelial cells failed to express galectin-1. Moreover, galectin-1 was expressed at the progressive stage of ICC. Up-regulation of galectin-1 expression in ICC cells was significantly correlated

**TABLE 5.** Relationship Between Biliary Epithelial Galectin-3 Expression and Pathologic Features in 40 Cases of ICC

	No. of cases	Galectin-3 Expression*			
		-	1+	2+	3+
Type					
Hilar	15	1	3	5	6
Peripheral	25	4	6	11	4
Size					
< 4 cm	19	1	5	5	8†
≥ 4 cm	21	4	4	11	2†
Perineural invasion					
Negative	24	1	4	11	8
Positive	16	4	5	5	2
Vascular invasion					
Negative	19	2	4	5	8
Positive	21	3	5	11	2
Lymphatic permeation					
Negative	13	1	4	2	6
Positive	27	4	5	14	4
Lymph node metastasis					
Negative	29	1	7	12	9†
Positive	11	4	2	4	1†

\* -, negative; 1+ <25% of cells positive; 2+, 25% to 65% positive; 3+, >65% positive.

† Mann-Whitney  $U$  test,  $P < .05$ .

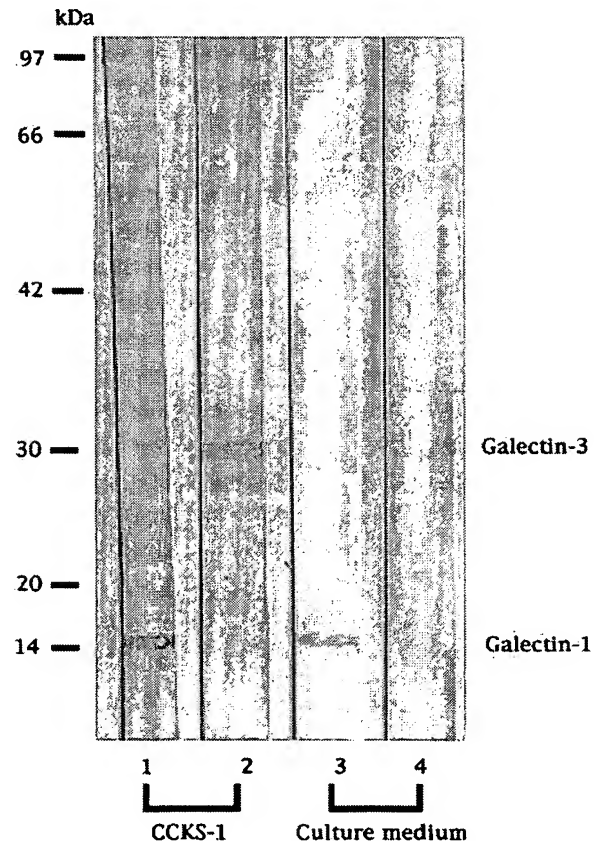


**FIGURE 3.** Expression of galectin-1 in the cytoplasm of cultured carcinoma cells. (Vector Red and hematoxylin, examined under a confocal microscope; original magnification  $\times 800$ .)

with histologic dedifferentiation of ICC and vascular invasion, lymphatic permeation, and lymph node metastasis of ICC. The carcinoma cell line CCKS1 was also found to express galectin-1. Although these findings suggest that galectin-1 expression is an indicator of the malignant potential of ICC and of dedifferentiated ICC, it remains unclear whether the expression of galectin-1 occurs as a result of transformation of biliary epithelial cells or galectin-1 induces malignant transformation. Considering that introduction of galectin-1 complementary DNA in BALB3T3 cells results in the acquisition of a transformed phenotype,<sup>17</sup> the latter possibility seems more plausible. Cindolo et al<sup>46</sup> also report that galectin-1 messenger RNA levels in the urinary bladder are markedly increased in high-grade tumors compared with normal bladder or low-grade tumors. Moreover, galectin-1 has been found both to promote and to inhibit cell proliferation.<sup>14-17</sup> For example, the expression of galectin-1 is correlated with increased proliferation in rat pulmonary arterial endothelial cells,<sup>16</sup> and Wells and Mallucci observed that exogenous galectin-1 inhibits the replication of mouse embryonic fibroblasts.<sup>15</sup> In the present study, the PCNA-LI was significantly higher in ICCs positive for galectin-1 than in those that were negative, suggesting that up-regulation of galectin-1 expression in ICC cells is correlated with proliferative activities in ICC.

This study also found that although connective tissue of portal tracts and parenchyma in normal livers was negative or weak for galectin-1, galectin-1 was intensively expressed in the cancerous stroma of ICC. In addition, the up-regulation of galectin-1 expression in stroma was significantly correlated with histologic dedifferentiation of ICC, and this up-regulation was significantly more common in ICCs with perineural and vascular invasion than in those without. The culture medium of cell line CCKS1 was found to contain galectin-1 as determined by Western blot analysis. These findings suggest that galectin-1 is produced and se-

creted by ICC cells and also possibly by stromal cells and that the galectin-1 secreted in ECM interacts with ICC cells and various stromal cells, contributing to the formation of a microenvironment favorable for cell growth and invasion of ICC. In other words, galectin-1 acts as an adhesion molecule such as integrins and cadherins in tumor progression through cell-cell and cell-ECM interaction. In fact, it has been reported in other carcinoma models that secreted galectin-1 mediates cell-cell and cell-ECM adhesion and promotes or inhibits the adhesion of cells to ECM.<sup>11,12,47</sup> Sanjuan et al<sup>24</sup> also report that colonic normal mucosa has a weak galectin-1 expression in both the epithelial and the stromal components, but increased stromal galectin-1 expression was observed at different stages of colorectal cancer progression. Galectin-1 was also expressed in the mesenchymal cells of the walls of stone-containing bile ducts, suggesting that galectin-1 is associated not only with neoplastic stroma formation but with fibrosis involving the bile ducts with inflammation. In hepatolithiasis, however, biliary epithelial cells were negative for galectin-1, suggesting that galectin-1 is secreted by mesenchymal cells.



**FIGURE 4.** Immunoblotting of galectin-1 and -3 in CCKS1 cells (lanes 1 and 2) and culture medium (lanes 3 and 4). CCKS1 cells expressed both 14-kd (galectin-1) and 30-kd (galectin-3) protein, whereas the culture medium expressed only 14-kd protein (galectin-1).

In contrast, galectin-3 was constitutively but weakly expressed in normal and hyperplastic intrahepatic bile ducts, while its expression was strong in biliary dysplasia. Furthermore, in ICCs, galectin-3 expression was more intense and widespread in well-differentiated ICC, particularly in areas with papillary and intraluminal growth, and was significantly decreased in dedifferentiated areas or poorly differentiated ICCs. Although up-regulation of galectin-3 has been reported to be associated with tumor progression in a variety of carcinomas, some reports suggest that galectin-3 is not associated with tumor-progressive factors. For example, Lotan et al<sup>27</sup> report that metastatic properties in gastric carcinomas do not correlate with galectin-3 levels. Van den Brule et al<sup>30</sup> failed to show a correlation between galectin-3 expression and clinicopathological features in ovarian carcinomas. The present study shows that expression of galectin-3 did not correlate with the occurrence of perineural and vascular invasion and lymphatic permeation. These findings suggest that galectin-3 expression cannot be considered as a marker of tumor progression in ICC, but galectin-3 expression is rather related to its preneoplastic and early neoplastic transformation. In other liver malignancies, Hsu et al<sup>32</sup> report that in human cirrhotic livers, proliferating hepatocytes expressing galectin-3 in regenerative nodules are in the process of being transformed, indicating an early neoplastic event. This galectin may be involved in the architectural preservation of ICC through its adhesive functions. It has been reported that in cell-cell and cell-ECM interaction, galectin-3 is essential for adhesion to laminin, the main basement membrane, and that galectin-3 may induce homotypic adhesion of tumor cells.<sup>9,10,13</sup> Down-regulation of galectin-3 expression was significantly correlated with tumor size (>4 cm) and lymph node metastasis. Therefore, decreased expression of galectin-3 may reflect the ability of ICC cells to detach from each other and the basement membrane, especially laminin, and this down-regulation may facilitate invasion and metastasis.

Galectin-3 has not been reported to directly regulate cell proliferation. However, Inohara et al<sup>14</sup> report that galectin-3 is a mitogen capable of stimulating fibroblast cell proliferation in a paracrine fashion through lectin-carbohydrate interaction. The second intron of the galectin-3 gene contains a promoter that is subject to down-regulation by the tumor-suppressor and cell cycle-regulatory protein p53.<sup>48</sup> Our study showed no significant correlation between galectin-3 expression and PCNA-LI in ICC, suggesting that the expression of galectin-3 is not associated with tumor cell proliferation in ICC. Lotz et al<sup>29</sup> report that the level of expression of galectin-3 is lower in colon carcinomas than in adenomas and normal colonic epithelium. Thus, galectin-3 may participate in the regulation of cell proliferation in nontransformed cells. This may also be the case in the biliary tree, because galectin-3 was more strongly expressed in the foci of biliary epithelial dysplasia with a higher PCNA-LI than in normal bile ducts in this study.

In conclusion, expression of galectin-1 and -3 is related to the progression and infiltration of ICC and to preneoplastic and early neoplastic changes of biliary epithelial cells, respectively. Particularly, the aberrant expression of galectin-1 in ICC acts to increase cell proliferation in ICC and tumor progression and acts as a factor in stromal formation.

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# Mac-2-Binding Protein Is a Diagnostic Marker for Biliary Tract Carcinoma

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**BACKGROUND.** Biliary tract carcinoma is a deadly disease, accounting for nearly 4500 malignancy-related deaths each year in the United States. Early detection has the potential to improve survival for patients with biliary tract malignancies, enabling curative surgical resection. Early detection approaches would benefit from an accurate, minimally invasive diagnostic test. To identify novel diagnostic markers, the authors recently completed a comprehensive proteomic study of bile samples from patients with biliary carcinoma. One of the proteins identified by tandem mass spectrometry was Mac-2-binding protein (Mac-2BP). The authors evaluated the performance of Mac-2BP and its ligand, galectin-3, as diagnostic markers for patients with biliary carcinoma.

**METHODS.** Levels of Mac-2BP, galectin-3, and CA19-9 were measured using an enzyme-linked immunosorbent assay (ELISA) in bile samples from patients with biliary tract carcinoma ( $n = 26$ ), benign biliary conditions ( $n = 32$ ), and primary sclerosing cholangitis ( $n = 20$ ). Serum levels of Mac-2BP and galectin-3 also were determined using ELISA. Mac-2BP tissue expression was investigated by immunohistochemical methods using a biliary carcinoma tissue microarray.

**RESULTS.** Biliary Mac-2BP levels were elevated by a factor of  $\sim 3$  in the biliary carcinoma group compared with the group of patients who had PSC or another type of nonneoplastic biliary disease. In contrast, Mac-2BP levels were not elevated in serum samples from patients with biliary carcinoma. According to the immunohistochemical analysis, Mac-2BP was expressed in 34 of 36 patients (94.4%) with biliary tract carcinoma. As a diagnostic marker for biliary carcinoma, Mac-2BP levels were as accurate as biliary CA19-9 levels, with an area under the curve (AUC) of 0.70 on receiver operator characteristic analysis. The use of both of these bile markers in combination, however, led to significantly better diagnostic accuracy compared with the accuracy achieved using CA19-9 alone (AUC, 0.75;  $P < 0.001$ ). Serum and biliary galectin-3 levels did not differ in the biliary carcinoma group relative to the control groups.

**CONCLUSIONS.** Biliary Mac-2BP levels, especially when used in conjunction with biliary CA19-9 levels, showed promise as a novel diagnostic marker for biliary tract carcinoma. *Cancer* 2004;101:1609–15. © 2004 American Cancer Society.

**KEYWORDS:** biliary tract carcinoma, cholangiocarcinoma, tumor marker, primary sclerosing cholangitis, Mac-2-binding protein, galectin-3.

The incidence of biliary tract carcinoma, which is defined as cholangiocellular carcinoma of the intrahepatic or extrahepatic bile duct or adenocarcinoma of the gallbladder, has increased sharply in recent decades in Western countries.<sup>1–3</sup> In the United States, an estimated 7500 new cases of carcinoma of the gallbladder or extrahepatic bile duct were diagnosed in 2003 alone, with nearly 4500 patients (65%) dying of disease.<sup>4</sup> The major risk factor for cholangiocellular carcinoma in Western countries is primary sclerosing cholan-

gitis (PSC), a chronic inflammatory disorder of the intrahepatic and extrahepatic bile ducts that carries a 10% lifetime risk of developing cholangiocarcinoma.<sup>5,6</sup> Currently, the only potentially curative therapy for bile duct carcinoma is complete surgical resection.<sup>7-9</sup> Curative surgical resection is associated with a 5-year survival rate of 9–18% for patients with proximal carcinomas, 20–30% for patients with distal extrahepatic bile duct tumors, and up to 40% for patients with intrahepatic cholangiocarcinomas.<sup>7</sup> Chemotherapy is largely ineffective against such malignancies, and there are no known treatments for PSC that reduce the risk of developing cholangiocarcinoma.<sup>7,9</sup>

Tumor stage, resectability, and survival are all closely correlated in patients with bile duct carcinoma, suggesting that early detection strategies may improve the prognosis of patients with this disease.<sup>7,10,11</sup> The potential survival benefit associated with early detection is demonstrated by survival data for patients with biliary carcinoma arising in the setting of PSC. The survival of patients with asymptomatic incidental biliary carcinoma that is discovered during liver transplantation for PSC is similar to the survival of patients who undergo transplantation in the absence of a biliary malignancy (70–80% at 5 years). In contrast, when PSC-associated biliary tract carcinoma is clinically apparent, prognosis is poor (0% at 5 years in the presence of lymph node metastases).<sup>12</sup>

Early detection efforts currently are hampered by the absence of a sufficiently accurate and noninvasive diagnostic test.<sup>11</sup> CA19-9 has been used as a marker for early-stage biliary carcinoma in patients with PSC; however, it has limited utility.<sup>13-18</sup> An alternative approach to diagnosing biliary tract carcinoma at an early stage involves searching for tumor markers in bile. Proteins derived from malignant cells of the biliary tree generally are present in greater concentrations in bile than in other body fluids. Several biliary markers, including CA19-9, carcinoembryonic antigen (CEA), CA125, and fibronectin, have shown promise in the diagnosis of cholangiocarcinoma.<sup>19-22</sup>

Recent advances in mass spectrometry allow comprehensive studies of malignancy-related changes in the proteome of body fluids to be performed.<sup>23-25</sup> We recently completed a proteomic study of human bile using a liquid chromatography–tandem mass spectrometry approach.<sup>26</sup> Because the bile samples used in that study were derived from patients with biliary tract carcinoma, several malignancy-related proteins were identified. Of these proteins, we chose Mac-2-binding protein (Mac-2BP; serum 90K) for further analysis, because this protein was detected in bile fluid using several different fractionation techniques and because

**TABLE 1**  
Bile and Serum Samples Used for Mac-2 Binding Protein Enzyme-Linked Immunosorbent Assay

Sample source	No. of patients		
	Biliary tract carcinoma	Benign biliary condition	Primary sclerosing cholangitis
Bile	26	32	20
Serum	28*	19	17

\* Includes 22 serum samples from patients with biliary carcinoma who underwent pancreatic duodenectomy; there were no matching bile samples.

there is evidence that it is expressed in several tumor entities.<sup>27-30</sup> Mac-2BP is a secreted glycoprotein that belongs to the scavenger receptor cysteine-rich domain protein family and originally was identified as tumor-associated antigen in breast carcinoma cells.<sup>31</sup> Mac-2BP plays a role in cell-cell and cell-matrix adhesion, binding to galectin-1, galectin-3, fibronectin, and collagens.<sup>32,33</sup> Elevated serum Mac-2BP levels have been detected in hepatocellular carcinomas and in pancreatic juice, and Mac-2BP levels are elevated in pancreatic adenocarcinoma.<sup>34,35</sup> In addition, a Mac-2BP ligand, galectin-3, also has been identified as a potential tumor marker.<sup>36</sup>

To evaluate the performance of Mac-2BP as a diagnostic marker for biliary tract carcinoma, we measured levels of Mac-2BP, galectin-3, and CA19-9 in bile using an enzyme-linked immunosorbent assay (ELISA); in addition, we measured serum levels of Mac-2BP and galectin-3. Samples were obtained from three distinct clinical subsets: patients with confirmed biliary tract carcinoma, patients with nonmalignant biliary disorders, and patients who had PSC without evidence of malignancy. Finally, we investigated tissue expression of Mac-2BP in biliary tract carcinomas using immunohistochemical methods.

## MATERIALS AND METHODS

### Bile and Serum Samples

Appropriate permission to perform the current study was obtained from the Johns Hopkins Joint Committee for Clinical Investigation. Bile samples were collected by endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic drainage (PTC) from patients with biliary tract carcinoma ( $n = 26$ ), patients with benign biliary conditions ( $n = 32$ ), and patients with PSC (a total of 20 samples obtained from 17 patients) (Table 1). In the group of patients with carcinoma, the malignancy was intrahepatic in six cases, located at the bifurcation (Klatskin tumor) in three cases, located in the proximal com-



mon bile duct in one case, located in the gallbladder in one case, located in the distal common bile duct in nine cases, and located in the ampulla in six cases. The TNM stage distribution was as follows: T2N0Mx,  $n = 6$ ; T2N1Mx,  $n = 2$ ; T2NxMx,  $n = 1$ ; T3N0Mx,  $n = 3$ ; T3N1Mx,  $n = 2$ ; and T4N0Mx,  $n = 1$ . Data were unavailable for 11 patients. The diagnosis of biliary tract carcinoma (all adenocarcinomas) was based on histologic examination in 20 patients and on cytologic examination in 6 patients with biliary carcinoma. The mean age of patients was  $65.5 \pm 14.1$  years in the carcinoma group and  $51.9 \pm 17.0$  years in the combined control group (benign conditions plus PSC). The clinical diagnoses for patients with benign biliary disorders included gallbladder or bile duct stones ( $n = 7$ ), pancreatitis ( $n = 8$ ), cirrhosis ( $n = 3$ ), cholecystitis/cholangitis ( $n = 2$ ), primary biliary cirrhosis ( $n = 4$ ), periampullary diverticula ( $n = 1$ ), and status post-liver transplantation ( $n = 1$ ). One patient who had a low-grade bile duct lymphoma also was included in this group. In the PSC group, of the 17 patients who had brush cytology performed to ascertain the presence of dysplasia, 1 patient had positive cytologic findings.

Matching serum samples were obtained from 6 of 26 patients with biliary tract carcinoma, from 19 of 32 patients with benign biliary conditions, and from 17 of 20 patients with PSC (Table 1). An additional 22 preoperative serum samples were obtained from patients with biliary carcinoma who underwent pancreaticoduodenectomy at The Johns Hopkins Medical Institutions (Baltimore, MD). Bile and serum samples were stored at  $-80^\circ\text{C}$  without additives, and all samples were subjected to fewer than 3 freeze-thaw cycles.

#### Mac-2BP, Galectin-3, and CA19-9 ELISA

Serum and bile samples were used for the Mac-2BP ELISA (BMS234; Bender MedSystems, San Bruno, CA), the galectin-3 ELISA (BMS279; Bender MedSystems), and the CA19-9 ELISA (catalog no. 1840; Alpha Diagnostics, San Antonio, TX), all of which were performed according to the manufacturers' specifications. The assays were performed using an ELx50 automated ELISA plate washer (BIO-TEK Instruments, Highland Park, VT), absorbance at 450 nm was read on an EL312e spectrophotometer (BIO-TEK Instruments), and results were calculated using the KC Junior software package (Version 1.40.3; BIO-TEK Instruments).

#### Mac-2BP Immunohistochemistry

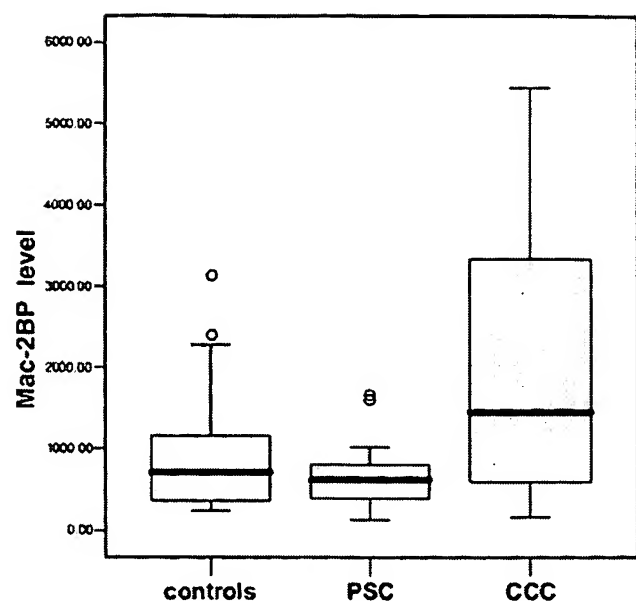
Formalin-fixed, paraffin-embedded biliary carcinoma tissue samples and control samples were obtained from the surgical pathology archives of the Department of Pathology at The Johns Hopkins Medical Institutions. Tissue microarrays were constructed as de-

scribed previously.<sup>37</sup> The biliary carcinoma tissue microarray included 38 adenocarcinoma samples (13 gallbladder, 10 intrahepatic, and 15 extrahepatic) and 2 small cell carcinomas of the gallbladder. For immunohistochemical analysis, sections were deparaffinized in xylene and graded ethanols, boiled for 30 minutes in 0.01 mM citrate buffer (antigen retrieval solution; DAKO, Carpinteria, CA), cooled for 20 minutes, rinsed with water, and washed for 5 minutes in wash buffer (TBS-T; DAKO). Endogenous peroxidases were blocked by incubation with 0.03%  $\text{H}_2\text{O}_2$  (peroxidase block; DAKO) followed by a wash buffer rinse and incubation overnight at  $4^\circ\text{C}$  with mouse anti-human monoclonal antibody 1A4.22 diluted 1:50 (antibody dilution buffer; DAKO). After a wash buffer rinse, link + biotin and streptavidin-horseradish peroxidase conjugate were applied for 15 minutes each (LSAB kit; DAKO), and sections subsequently were developed with diaminobenzidine, counterstained with hematoxylin, dehydrated, and mounted. Immunohistochemical staining intensity and staining area were assessed by two observers (A.M. and J.K.). Staining intensity was scored as *negative*, *mild*, *moderate*, or *strong*; staining area was scored as *negative* ( $< 1\%$  of all neoplastic cells), *focal positive* (1–25% of all neoplastic cells), or *diffuse positive* (26–100% of all neoplastic cells). Overall staining was considered to be positive when at least mild staining intensity could be demonstrated in  $> 25\%$  of all tumor cells.

#### Statistical Analysis

The major statistical endpoint in the current study was the receiver operator characteristic (ROC) analysis of biliary CA-19 and Mac-2BP levels in the diagnosis of biliary tract carcinoma. The area under the ROC curve (AUC) was calculated for each marker, and bootstrap bias-corrected accelerated confidence intervals were computed using Version 2.5 of the AccuROC software package (Accumetric Corp., Montreal, Quebec, Canada). Comparisons of these correlated single-marker curves were made with AccuROC software using the method described by DeLong et al.<sup>38</sup> To compare the predictive value of a model that used both markers with the value of a model that used CA-19 alone, a bootstrap approach was taken.<sup>39</sup> Bootstrap samples were used to estimate the AUC for each model and to estimate differences. Significance was determined using a paired Student  $t$  test. Mean values were compared using logarithmic transformation of the data for Mac-2BP levels and using a square-root transformation for CA19-9 levels. Statistical computations were performed using AccuROC software (Accumetric Corp.) or the SAS system (SAS Institute, Cary, NC).



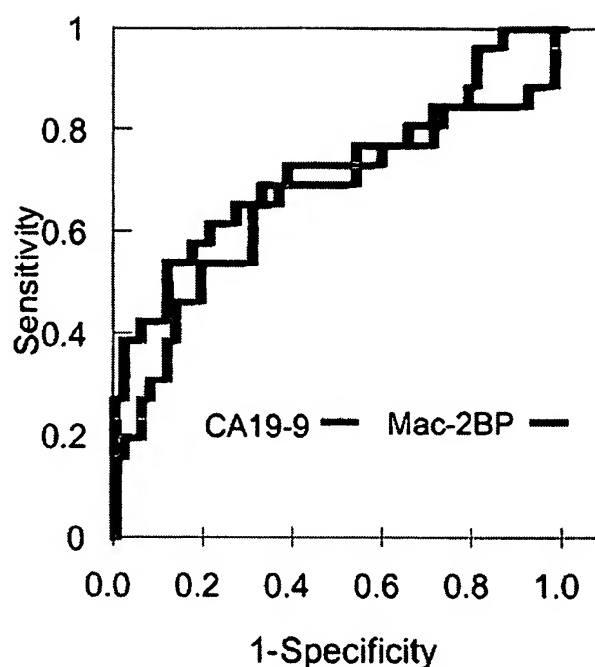


**FIGURE 1.** Mac-2-binding protein (Mac-2BP) levels in bile from patients with biliary carcinoma, bile from patients with benign biliary conditions, and bile from patients with primary sclerosing cholangitis (PSC). CCC: cholangiocarcinoma.

## RESULTS

### Mac-2BP, CA19-9, and Galectin-3 Levels in Bile

Mac-2BP, CA19-9, and Galectin-3 levels were measured by ELISA in bile samples from 26 patients with biliary tract carcinoma, in samples from 32 patients with benign biliary conditions, and in a total of 20 samples from 17 patients with PSC. The mean ( $\pm$  standard deviation [SD]) biliary Mac-2BP level was  $2081 \pm 1757$  ng/mL in the biliary tract carcinoma group,  $879 \pm 679$  ng/mL in the benign biliary condition group,  $656 \pm 415$  ng/mL in the PSC group, and  $793 \pm 694$  ng/mL in both control groups (benign biliary conditions plus PSC) combined (Fig. 1). All differences in Mac-2BP levels between the carcinoma group and the individual control groups and between the carcinoma group and the combined control group were statistically significant ( $P < 0.05$ ). No significant differences were found in terms of biliary Mac-2BP levels when samples associated with different sources (ERCP vs. PTC) and different tumor histologies (gallbladder carcinoma vs. cholangiocarcinoma) were compared. The mean ( $\pm$  SD) biliary CA19-9 level was  $57,343 \pm 39,855$  units (U) per mL in the biliary carcinoma group,  $29,130 \pm 26,614$  U/mL in the benign biliary condition group,  $34,466 \pm 38,066$  U/mL in the PSC group, and  $31,182 \pm 3,126$  U/mL in the combined control group. The difference in CA19-9 levels between carcinoma samples and combined control



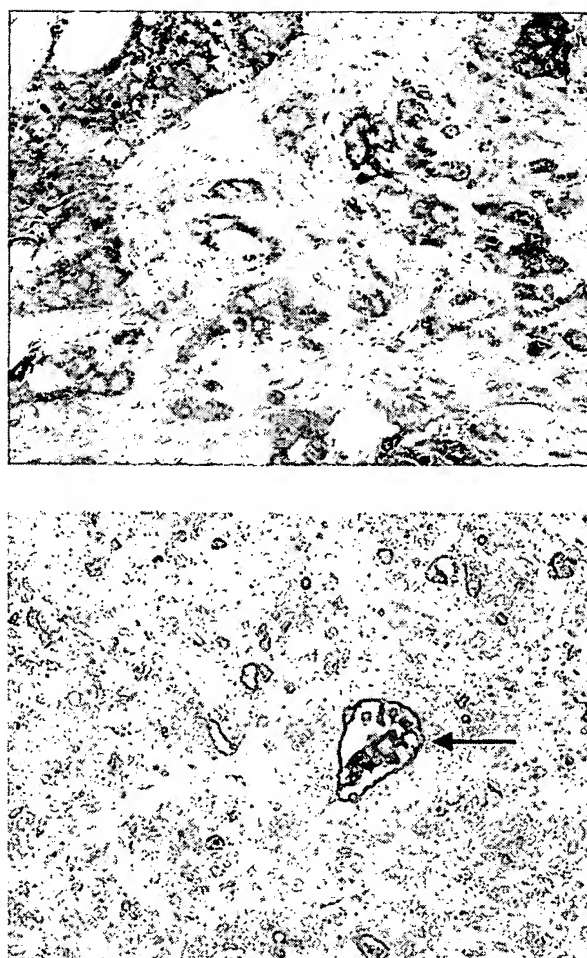
**FIGURE 2.** Receiver operating characteristic analysis of bile Mac-2-binding protein (Mac-2BP) and CA19-9 levels.

group samples was significant ( $P < 0.05$ ). CA19-9 levels in carcinoma samples and PSC samples were not statistically significantly different from each other. Galectin-3 levels in bile were not significantly different in the carcinoma group relative to the combined control group (mean  $\pm$  SD:  $56.2 \pm 39.9$  ng/mL vs.  $52.6 \pm 33.8$  ng/mL, respectively).

### Evaluation of Biliary Mac-2BP and CA19-9 Levels as a Diagnostic Test

The performance of Mac-2BP and CA19-9 levels in bile as diagnostic markers was evaluated using receiver operating characteristic (ROC) analysis. The AUC was 0.70 (95% confidence interval [CI], 0.54–0.83) for Mac-2BP and was almost identical for CA19-9 (0.69; 95% CI, 0.52–0.81) (Fig. 2). Combining both markers improved their diagnostic utility, yielding an AUC of 0.75 (95% CI, 0.65–0.89), significantly higher than the AUC associated with the use of CA19-9 as a single marker ( $P < 0.001$ ).

Using an optimized cutoff point derived from the ROC curve (853 ng/mL), biliary Mac-2BP as a single marker had sensitivity of 0.69 and specificity of 0.67 in the diagnosis of biliary tract carcinoma. The corresponding sensitivity of CA19-9 (cutoff point, 36,550 U/mL) was 65% at 65% specificity.



**FIGURE 3.** Immunohistochemical analysis of Mac-2-binding protein (Mac-2BP) in biliary carcinoma tissue. (A) Cholangiocarcinoma with strong cytoplasmic labeling of Mac-2BP. (B) Cholangiocarcinoma with luminal accentuation of Mac-2BP staining (arrow) and Mac-2BP-positive material in lumen of glandular structures. Original magnification  $\times 100$  (A);  $\times 64$  (B).

#### Serum Mac-2BP and Galectin-3 Levels

Serum levels of Mac-2BP and galectin-3 were determined by ELISA in 64 patients (28 patients with biliary carcinoma, 19 patients with benign biliary conditions, and 17 patients with PSC). Mac-2BP and galectin-3 levels in serum did not differ significantly in the carcinoma group relative to the combined control group (mean  $\pm$  SD: Mac-2BP,  $8036 \pm 5707$  ng/mL vs.  $8782 \pm 3943$  ng/mL; galectin-3,  $7.4 \pm 3.8$  ng/mL vs.  $7.2 \pm 5.9$  ng/mL).

#### Mac-2BP Immunohistochemistry

Overall, 34 of 36 tumor tissue cores (94.4%) exhibited positive cytoplasmic staining for Mac-2BP (Fig. 3A). Twelve of those cores (33.3%) exhibited mild immu-

nohistochemical labeling, 9 cores (28%) exhibited moderate immunohistochemical labeling, and 13 cores (36.1%) exhibited strong immunohistochemical labeling. The staining area was diffusely positive ( $> 25\%$  of all tumor cells) in all 34 positive specimens. The 2 tumor specimens on the array that were negative for Mac-2BP staining were obtained from small cell carcinomas of the gallbladder, whereas all 34 positive specimens were obtained from adenocarcinomas. Staining of tumor cell epithelia revealed frequent accentuation at the luminal borders of neoplastic glands (Fig. 3B). Within these glandular structures, intraluminal material, presumably secreted protein, also stained positively for Mac-2BP. Specific staining was not seen in the peritumoral stroma or in macrophages, and a variety of control cores that were included in the tissue microarray, such as normal pancreatic and biliary ductal structures and gallbladder epithelium, were negative for Mac-2BP immunolabeling as well. Normal pancreatic control tissue exhibited strong positive staining of islet cells, as has been reported previously (data not shown).<sup>30</sup>

#### DISCUSSION

In the current study, we evaluated the measurement of Mac-2BP levels in bile as a novel diagnostic test for biliary tract carcinoma. Bile Mac-2BP levels were elevated by a factor of  $\sim 3$  in samples obtained from patients with biliary carcinoma compared with samples obtained from patients with benign biliary conditions and from patients with PSC without evidence of malignancy. The diagnostic performance of biliary Mac-2BP as a single marker was comparable to that of biliary CA19-9 (AUC values for ROC analysis, 0.70 and 0.69, respectively). When biliary Mac-2BP was used in conjunction with CA19-9, however, the resulting model had significantly better diagnostic performance than did the model that used CA19-9 alone (AUC, 0.75). The diagnostic accuracy of Mac-2BP as single marker compared favorably with the performance of other protein markers for patients with biliary tract carcinoma (specifically, CEA, CA125, CA19-9, and fibronectin), which have reported sensitivities of 57–70% and specificities of 33–79%.<sup>19–22</sup> In addition, with respect to sensitivity, our combined model outperformed the current histopathologic gold standard, biliary brush cytology performed in conjunction with biopsy, which has a sensitivity of only 30–44%.<sup>7,40</sup> To date, nucleic acid-based tests that use bile samples to diagnose biliary malignancies (e.g., *K-ras* mutation analysis and telomerase reverse transcriptase mRNA amplification) also have yielded lower levels of diagnostic sensitivity compared with the current combined model.<sup>41–43</sup>

Due to the limited nature of the current sample set, further study will be needed to define the role of Mac-2BP in the detection of PSC-associated dysplasia. It is possible that serial measurements of markers such as Mac-2BP and CA19-9 may provide more information regarding the presence of PSC-associated neoplasia. In this regard, it is noteworthy that both bile samples from the lone patient with PSC who had severe dysplasia on brush cytology exhibited Mac-2BP and CA19-9 levels that were higher than the selected cutoff levels (Mac2B, 1614 ng/mL; CA19-9, 36,558 U/mL).

In contrast to bile Mac-2BP levels, serum levels of Mac-2BP did not distinguish the carcinoma group from the control groups. A possible explanation for this discrepancy is the up-regulation of serum Mac-2BP in response to a variety of nonmalignant inflammatory conditions,<sup>34,44</sup> obscuring the increase in Mac-2BP levels in a tumor that typically has a small mass. This underscores the value of using a bodily fluid with direct access to tumor cells—in this instance, bile—as diagnostic material. Most patients with suspected biliary conditions, and particularly patients with PSC or suspected cholangiocarcinoma, routinely undergo diagnostic ERCP. These patients undergo brush cytologic examination to identify neoplasia and could have endoscopic bile samples obtained without additional clinical intervention if a suitable marker panel were available.

In addition to Mac-2BP and CA19-9, we also analyzed bile and serum levels of galectin-3 as potential markers of biliary malignancy. Galectin-3 is a ligand for Mac-2BP, and galectin-3 protein expression has been reported previously in intrahepatic cholangiocellular carcinoma.<sup>31</sup> However, the use of bile and serum galectin-3 levels in the current study did not improve diagnostic capabilities. It is noteworthy that we did not identify galectin-3 in our initial proteomic analysis of bile from patients with biliary carcinoma (unpublished data). The value of proteomic profiling as a diagnostic tool is highlighted by the finding that Mac-2BP mRNA levels were not elevated by a factor of > 3 in a recent study of biliary carcinoma gene expression using oligonucleotide microarrays,<sup>45</sup> demonstrating that not all overexpressed proteins will be identified using gene expression approaches.

In conclusion, we have demonstrated that Mac-2BP levels in bile represent a promising tumor marker for the diagnosis of biliary tract carcinomas, especially when these levels are used in combination with biliary CA19-9 levels. Further studies involving a larger patient population will be needed to provide more information regarding the diagnostic accuracy of Mac-2BP, particularly in patients who have a high risk of

developing biliary carcinoma (e.g., those with PSC), so that it can be determined whether a panel of markers can be used to detect biliary malignancies at an early (and thus potentially curable) stage.

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